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Epigenetics of Aberrant Cardiac Wound Healing

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Abstract

Remodeling of cardiac tissue architecture is essential for normal organ development and maintaining homeostasis after injury. Injurious insults to the heart, such as hypertension and myocardial infarction, promote cellular responses including stimulation of resident inflammatory cells, activation of endothelial cells and recruitment of immune cells, hypertrophy of cardiomyocytes and activation of fibroblasts. The physiological goal of this coordinated cellular response is to repair damaged tissue whilst maintaining or restoring cardiac contractile function. Persistent uncontrolled inflammation, hypertrophy and fibrosis in the heart due to hyperactive wound healing are detrimental and impair cardiac performance, facilitating the progression to heart failure. Abnormal changes in gene expression promote acquisition of aberrant cellular phenotypes that drive cardiac remodeling. DNA methylation and histone modifications are epigenetic mechanisms that critically regulate chromatin structure and gene expression, and are essential for normal physiology and development. Increasing clinical and experimental evidence suggests that these epigenetic mechanisms are involved in driving aberrant wound healing and the development of heart failure. While most of our knowledge to date is on the heart as a whole, the precise contribution of DNA methylation and histone modifications in regulating aberrant cardiac remodeling at the cellular level is less defined. Therefore, this overview aims to summarize the role of DNA methylation and histone modifications (acetylation and methylation) in heart failure and to comprehensively dissect the role these mechanisms play in regulating the function of cardiomyocytes, fibroblasts and immune cells in response to injury.

Didactic Synopsis

Cardiac remodeling is a crucial feature of myocardial responses to injury. Epigenetic alterations and regulation of this multifactorial process are at the forefront of cardiac remodeling research, including the delineating of cell-type specific contributions. This comprehensive review will support undergraduate and postgraduate teaching of concepts relating to the role of epigenetics in dictating inflammatory, fibrotic, and hypertrophic responses in the context of cardiac injury.

Major Teaching Points:

1) Understanding wound healing responses of both resident cardiac cells and infiltrating inflammatory cells, in pathological settings such as myocardial infarction and pressure overload, is necessary to appreciate the complex pathophysiology that leads to the development of heart failure.
2) Epigenetic mechanisms such as DNA methylation and post-translational modifications (acetylation and methylation) of histone tails are important regulators of gene expression and

are now considered to be involved in promoting abnormal cardiac wound healing through, extracellular matrix deposition, myocyte hypertrophy and inflammatory mediator release.

3) Epigenetic mechanisms critically regulate cellular phenotype and function;
   a) Alterations in DNA methylation (e.g. DNMT3 enzymes) and histone modifications (e.g. HDAC2) can influence the hypertrophic response in post-mitotic cardiomyocytes.
   b) Increased DNA methylation in cardiac fibroblasts is associated with sustained activation and extracellular matrix deposition.
   c) Immune cell phenotype, cytokine release and immune response (trained immunity vs immunotolerance) are regulated by both DNA methylation and histone modifications.
4) Advancements in therapeutic targeting or manipulation of the epigenetic machinery in cells implicated in aberrant cardiac remodeling may yield novel treatment strategies in the future management of cardiac diseases including heart failure.

Introduction

Heart failure is a complex disorder that is one of the leading global causes of morbidity and mortality, and is associated with a significant economic cost (129, 231). It is the most common cause of hospitalization in patients aged 65 years and over (183) and while therapeutic advances have been encouraging (231), the outlook for patients with heart failure still remains a concern as roughly half of all patients die within 5 years of diagnosis (107).

The progression to cardiac failure begins with pathological insults to the heart. These insults can be acute, as in the case of an ischemic injury due to myocardial infarction or chronic damage inflicted as a result of hypertension (HTN), metabolic dysfunction (diabetes and obesity), inflammatory disease (myocarditis), toxins (alcohol, cytotoxic drugs), genetic mutations, valvular disease and prolonged arrhythmias (66, 166, 319). Regardless of the origin, injury to the heart evokes a diverse and complex array of cellular responses involving cardiomyocytes and non-muscle cells, including fibroblasts, endothelial cells and immune cells in order to repair damaged tissue and remodel the myocardium in order to maintain cardiac function (37, 42, 176). Persistent injury or exaggerated wound healing responses can lead to aberrant cardiac tissue remodeling that impacts on cardiac function. This remodeling of the heart, which manifests clinically as changes in the size, shape and function of the heart, is now accepted as a key determinant of the clinical course of heart failure (66). Whilst different types of injury activate overlapping cellular responses, the pathophysiological consequences can be quite different. For example, myocardial infarction induces myocyte death in the ischemic region that is accompanied by inflammation and reactive fibrosis (scar formation). Reduced cardiac contractility leads to systolic dysfunction and heart failure with reduced ejection fraction. On the other hand, chronic hypertension (increased cardiac afterload) leads to inflammation, interstitial fibrosis and myocyte hypertrophy, ultimately leading to reduced cardiac compliance, diastolic dysfunction and heart failure with preserved ejection fraction. Understanding what regulates cardiac cell adaptation to injury requires careful consideration of the nature of the injurious insult, the tissue microenvironment, and the cell types involved.

Whilst much work has focused on understanding the role of specific transcription factors in regulating transcription from response elements in the promoters of specific disease-modifying genes, it is now known that epigenetic control of chromatin structure and transcriptional machinery plays a crucial role in shaping both individual and global gene expression patterns and regulating sustained changes in cellular phenotypes. Epigenetics is the heritable modulation of gene activity that is independent of the underlying DNA sequence. Epigenetic modifications include DNA methylation, histone modifications (e.g. acetylation and methylation) and non-coding RNAs (e.g. micro RNAs and long non-coding RNAs) (138, 149). Epigenetic modifications are fundamental for early development along with participating in various biological processes such as cellular proliferation and sustaining cellular identity (26, 221). Alterations to these epigenetic regulatory mechanisms have been implicated in numerous disease pathologies including imprinting disorders, cancer, neurodegenerative disease and cardiovascular disease (84, 232,

261). Therefore, it is no surprise that epigenetic alterations in response to acute or chronic injury may participate in driving aberrant cardiac wound healing.

A major issue with our understanding of epigenetic alterations in cardiac disease is that changes have largely been assessed in whole heart tissue, which contains a mixed cell population of cardiomyocytes, fibroblasts, endothelial cells, progenitor cells and immune cells. This makes it very difficult to distinguish which cells these pathologically-driven epigenetic changes are occurring in. Given the emerging evidence that epigenetic therapy has a beneficial impact in animal models of heart failure it is becoming increasingly important that we understand the mechanisms by which such therapies modulate cardiac cell function and the cardiac response to injury. This overview will focus on the impact of the epigenetic modifications, DNA methylation and histone modifications (acetylation and methylation), in the cellular effectors of cardiac remodeling and aberrant wound healing, focusing specifically on cardiomyocytes, fibroblasts and immune cells **(Figure 1)**. For a full list of abbreviations please see **Table 1.**

Aberrant Cardiac Wound Healing and Heart Failure

The use of various animal models of heart failure and human clinical samples has allowed us to dissect out the pathophysiological events that drive cardiac wound healing and tissue remodeling subsequent to cardiac injury. The initial insult to the heart, results in cellular death, tissue hypoxia, local cytokine production and generation of reactive oxygen species (ROS). These drivers promote immune cell infiltration to resolve tissue damage and to initiate wound healing in the heart. Cells of both the innate and adaptive immune response are implicated, including neutrophils, macrophages, monocytes, dendritic cells, B and T cells (86, 100, 301). These infiltrating immune cells release inflammatory mediators (tumor necrosis factor (TNF)-alpha, interleukin (IL) 1, IL-8, IL-6, IL-18, macrophage inflammatory protein (MIP)-1α, monocyte chemoattractant protein-1 (MCP-1), interferon (IFN)-γ, and transforming growth factor (TGF)-β) (36, 40, 95, 118, 327, 370). Both inflammatory mediators and neurohormonal signals (catecholamines, the renin-angiotensin-aldosterone system (RAAS) and endothelin) that are circulating and released locally by resident and infiltrating cells in combination with increasing tissue hypoxia and ROS generation have numerous effects that potentiate cardiac remodeling. These include promoting fibroblast proliferation and transdifferentiation to myofibroblasts, increased production and secretion of extracellular matrix (ECM) components (collagens I, collagen III, and fibronectin), endothelial dysfunction, hypertrophy of cardiomyocytes, further oxidative damage and cellular death (necrosis or apoptosis) (14, 307, 317). The long term effects of chronic inflammation and persistent aberrant remodeling within the heart are detrimental to cardiac function. In the setting of acute injury, such as myocardial infarction, exaggerated inflammation causes a weakening of scar tissue contributing to ventricular dilatation and systolic heart failure. In the setting of pressure overload chronic inflammation drives exaggerated wound healing responses and interstitial fibrosis that contributes to decreased compliance and diastolic dysfunction. As 75% of heart failure patients have antecedent hypertension (231) it is likely that many that suffer myocardial infarction already have evidence of pressure-overload related interstitial fibrosis and ventricular hypertrophy.

Cardiac hypertrophy involves the enlargement of myocytes in order to reduce ventricular and septal wall stress and maintain stroke volume by increasing contractile capacity (165). The hypertrophic response can be induced in physiological situations, such as exercise and pregnancy. However in these situations the hypertrophy of myocytes is finely tuned and mirrored with a consistent supporting vascular network with no evidence of accompanying cardiac fibrosis (190, 247).These features are not observed in the pathological setting; sustained myocyte hypertrophy becomes maladaptive and is positively associated with the development of heart failure. These defective changes in the cardiomyocyte include alterations in energy metabolism, cytoskeletal structure, myofilament function along with abnormal calcium regulation and excitation-contraction coupling (140, 174, 187, 319).

In many pathological settings cardiomyocyte hypertrophy is accompanied by increased ECM deposition, presumably with the goal of providing a more rigid scaffold to cardiac myocytes.

Production and enzymatic degradation of ECM is a finely controlled process, through matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs), and is essential for maintaining normal tissue architecture, especially after tissue injury. The molecular composition of the cardiac ECM and the cells and enzymes that are involved in ECM turnover have been comprehensively reviewed elsewhere (196, 320). Myofibroblasts are a principal cellular element involved in cardiac wound healing. At the site of injury, these mesenchymal cells (that express α-smooth muscle actin, αSMA) become activated, produce and secrete ECM proteins and have the ability to contract in order to facilitate wound healing (134, 285, 295).

In early wound healing responses, degradation of ECM components by inflammatory cell-derived MMPs liberates bioactive fragments known as matrikines that promote the inflammatory and reparative cascades. The formation of a provisional matrix derived from extravasated plasma proteins facilitates entry of inflammatory cells that promote clearance of necrotic tissue and the migration of fibroblasts that transdifferentiate into myofibroblasts. Early deposition of provisional matrix proteins, such as fibronectin, by fibroblasts is followed by the deposition of structural collagens by activated myofibroblasts (reviewed in (98)). During the maturation phase of wound healing disorganized thin elastic collagen III fibers gradually become replaced with organized thicker collagen I fibers that become increasingly cross-linked by lysyl oxidase enzymes and increase the tensile strength of the ECM (158). The relative contribution of type I and type III collagen fibers and the degree of cross-linking can have a significant impact on the mechanical properties of the ECM (68). While the exact origin of myofibroblasts in the heart remains controversial (134), the generally accepted hypothesis is that resident fibroblasts within the cardiac interstitium are prompted by environmental stimuli to proliferate and transdifferentiate into myofibroblasts. During the resolution of normal tissue repair processes, active myofibroblasts revert back to either quiescent fibroblasts or undergo cellular apoptosis to allow for proficient wound healing (81, 101). Disruption of this physiological process, either through overactive ECM production due to persistent hyperactive myofibroblast phenotype (156) or through defects in ECM degradation (185), results in pathological accumulation of ECM proteins, resulting in tissue fibrosis. Fibrosis within a dynamic organ such as the heart can have adverse effects causing abnormal tissue stiffness, reduced myocardial perfusion, defective electrical signalling and arrhythmias along with impaired cardiac contraction and relaxation (88, 176, 220).

In response to injurious insults, modulation of gene expression in tissue resident or infiltrating cells is essential to facilitate wound healing and appropriate myocardial remodeling (35, 117, 128). Considering heart failure has such a complex etiology, including a genetic and environmental input, it is crucial that we understand the mechanisms that regulate gene expression. This is particularly important as alterations in these mechanisms could be contributing to the sustained aberrant cellular phenotype (i.e. pro-hypertrophic, pro-fibrotic and pro-inflammatory phenotype) of cells that are implicated in driving pathological remodeling and promoting heart failure (**Figure 1**). In this regard some clues are provided by studies of cardiac development. Modulation of gene expression is crucial during normal cardiac development and it is of particular interest that during myocardial disease the damaged heart undergoes re-induction of the fetal gene program including re-expression of natriuretic factors (Nppa and Nppb), beta (β)-myosin heavy chain (Myh7) and alpha-skeletal actin (63). These data suggest that aberrant wound-healing may involve cell type-specific alterations at the level of gene-specific transcription factors that occur on top of epigenetic mechanisms such as DNA methylation and histone modification that influence global gene expression.

DNA Methylation

DNA methylation is a cellular process that involves the covalent attachment of a methyl residue to the carbon five prime position on a cytosine ring resulting in the formation of 5-methylcytosine (5MeC). This process mainly occurs in palindromic cytosine-(phosphate)-guanine dinucleotides (CpGs) throughout the genome, with the majority (60-80%) of these in somatic cells being methylated (303). Roughly 10% of CpGs are clustered together in regions known as CpG islands (CpGi's) and are associated with approximately 70% of annotated mammalian gene promoters

(287). These CpGi's are GC rich, roughly a thousand base pairs long and generally are unmethylated, facilitating transcriptional activity (78). CpGi's are flanked by CpG shores and shelves, which are < 2 kb or > 2 kb from CpGi's respectively and generally display dynamic changes in DNA methylation. Three DNA methyltransferase (DNMT) enzymes (DNMT1, DNMT3A and DNMT3B) are involved in methylating DNA, using the methyl donor, S-adenosylmethionine (SAM) to form 5MeC. DNMT1 is involved in the maintenance of established DNA methylation (294) whereas DNMT3A and DNMT3B carry out *de novo* DNA methylation (246). DNA methylation regulates gene expression through transcriptional repression both directly and indirectly. DNA methylation can directly interfere with transcription factor binding to recognition sites in the promoter region (72) (**Figure 2**). Indirect inhibition of gene expression can result through the recruitment of methyl-CpG-binding proteins such as methyl-CpG-binding protein (MeCP) 1 and 2 and methyl-CpG-binding domain protein (MBD) 1, 2, 3 and 4. Recruitment of these proteins promotes the interaction of co-repressor complexes with methylated DNA through their methyl-CpG-binding domain motif, leading to a transcriptionally-repressed chromatin state and subsequent gene silencing (72, 132).

While it was initially considered to be a persistent stable epigenetic mark, it is now apparent that DNA methylation is more dynamic in nature and can be reversed. Removal of DNA methylation can occur through oxidation of 5MeC to 5-hydroxymethylcytosine (5hMeC) which is facilitated by the ten-eleven translocation (TET) family of enzymes (TET1, TET2 and TET3) (**Figure 2**). The methylation mark is removed from the cytosine as 5hMeC undergoes deamination and subsequent base excision repair by thymine DNA glycosylase (173). Emerging evidence is suggesting that rather than being an intermediate product of DNA demethylation, 5hMeC may itself function to regulate gene expression (54, 362). Development of techniques such as isotope-based liquid chromatography mass spectrometry, glucosyl tagging and TET-assisted bisulfite sequencing (TAB-seq) has enabled researchers to determine the absolute levels and enrichments of 5hMeC within cells (255, 382).

Intragenic methylation within the gene body and transcriptional regions is involved in gene regulation and alternative splicing (131). The exact functional contribution that methylation of gene bodies has on governing gene expression is unclear with varying results reported within the literature. Some reports highlight increased transcriptional activity that is associated with hypermethylation within the gene body (19, 93, 269, 378), whereas hypomethylation is accompanied with reduced gene expression (297). Certain studies demonstrate the opposite, where increased gene expression is mirrored by demethylation of gene bodies (109, 194). The exact relationship between gene body methylation and gene expression is not fully understood; however its role in modulating gene expression could have different consequences in different cellular contexts (194, 214).

DNA Methylation in Heart Failure

Involvement of DNA methylation in the failing heart is becoming increasingly apparent (**Table 2**). Analysis of whole genome bisulfite sequencing (WGBS) data sets across human cell and tissue types has identified tissue-specific differentially methylated regions (397). Furthermore, regions with cardiac-specific DNA methylation patterns were shown to be enriched for single nucleotide polymorphisms (SNPs) linked to cardiovascular diseases in genome-wide association studies (397). Numerous preliminary studies are demonstrating alterations in DNA methylation within cardiac tissue from patients with chronic heart failure. Movassagh *et al*. investigated the global methylation profiles in cardiac tissue from a small cohort of patients with end stage heart failure (n=16), comparing them to non-diseased hearts (n=8) (230). They found that there was a global reduction in CpGi methylation at promoter regions which in parallel also showed increased methylation at intragenic regions in patients with cardiomyopathy. This was shown to correlate with areas of increased transcriptional activity. Genes that were globally down-regulated in patients with cardiomyopathy were not found to be significantly correlated to increased methylation at the promoter regions. One gene of interest that was found to have an enrichment of CpGi methylation in cardiac disease versus controls was the transcriptional activator *Dux4*. Hypermethylation of the *Dux4* locus corresponded to a downregulation in its expression in failing hearts (230). Previous work carried out by the same research group, using immunoprecipitation

of methylated DNA followed by methylation array analysis, demonstrated differential methylation at three gene loci (platelet endothelial cell adhesion molecule 1 (PECAM), Rho GTPase activating protein 24 (ARHGAP24) and angiomotin-like protein 2 (AMOTL2)), implicated in angiogenesis, in patients with end stage cardiomyopathy (229). Differential methylation was shown to correlate with gene expression with reduced expression of PECAM as a result of promoter methylation, increased gene expression due to AMOTL2 promoter hypomethylation and hypermethylation of the ARHGAP24 gene body (229).

Genome wide assessment of methylation profiles has also indicated aberrant CpGI methylation differences between idiopathic dilated cardiomyopathy (DCM) patients and controls (121). The study highlighted two genes of interest, LY75 (CpGi hypermethylation) and ADORA2A (CpGi hypomethylation), where altered CpGi methylation was correlated with an abnormal reduction in gene expression (121). *In silico* analysis revealed that CpGi hypomethylation in the ADORA2A gene promoter region is associated with the binding site for the transcriptional repressor CTCF, which could potentially account for why hypomethylation resulted in reduced gene expression. *In vivo* validation of reduced expression of these genes in a zebrafish model showed development of cardiac dysfunction and heart failure (121). Alterations of DNA methylation levels have also been reported in ischemic heart disease. Hypomethylation of long interspersed nuclear element-1 (LINE-1), a highly repeated and widely interspersed human retrotransposon) in mixed cell populations from patient blood samples was associated with ischemic heart disease and stroke (16). LINE-1 elements were also found to be hypomethylated in patients with the congenital heart defect Tetralogy of Fallot (299) and while not found to be significantly correlated, patients with Tetralogy of Fallot displayed reduced expression of the DNA methylating enzymes DNMT1, DNMT3A and DNMT3B (298). These LINE-1 elements are usually methylated in the majority of normal tissues (51).

Global changes in DNA methylation have also been shown to play a role in the progression of risk factors that contribute to heart failure development (390) such as hypertension (304, 341, 360)), obesity and type 2 diabetes (T2DM) (74, 180, 336, 368), and atherosclerosis (49, 133). In the context of hypertension, a recent genome-wide association and replication study by Kato *et al.*, involving 320,251 people of East Asian, European and South Asian origin, identified genetic variants at 12 new loci to be associated with blood pressure phenotype (162). They identified variants in genes involved in both vascular smooth muscle (IGFBP3, KCNK3, PDE3A and PRDM6) and renal (ARHGAP24 (previously mentioned above), OSR1, SLC22A7 and TBX2) function that are associated with methylation at multiple CpG sites, using both a 450K methylation array and targeted bisulfite sequencing. These genetic variants identified along with previously known variants were shown to predict increased left ventricular mass, circulating levels of N-terminal pro b-type natriuretic peptide (NT-proBNP), and both cardiovascular and all-cause mortality (162). This study indicates that DNA methylation may be the cellular process that links sequence variation and blood pressure regulation, suggesting that aberrant changes in DNA methylation can have pathological consequences for the heart.

Pharmacological Inhibition of DNA Methylation

Another aspect that highlights the involvement of DNA methylation in aberrant cardiac remodeling has been revealed through studies showing the therapeutic benefits of DNMT inhibitors in preclinical models of heart failure. 5-azacytidine (5aza or Vidaza) and its more potent deoxy derivative, 5-aza-2-deoxycytidine (5azadC or Decitabine), are DNMT inhibitors that are approved by both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of myelodysplastic syndromes (MDS) and Acute Myeloid Leukaemia (237). These demethylating agents are cytosine analogues and act by incorporating into DNA, and also RNA in the case of 5aza, forming tight covalent complexes with DNMT enzymes preventing their enzymatic activity and promoting their degradation (113). 5aza has previously been shown to have both anti-hypertrophic and anti-fibrotic actions in the Spontaneously Hypertensive Rat (SHR) model of HTN-induced heart failure (351). Reduction of pathological remodeling after treatment with 5aza associated with an improvement in cardiac structure and function, measured

by reduced LV mass and improved ejection fraction, compared to untreated SHR's (351). Another study assessing the effect of 5azadC in rats with norepinephrine-induced cardiac injury demonstrated that treatment with 5azadC reduced catecholamine-induced DNA hypermethylation (363). By Langendorff preparation of the isolated hearts, the authors demonstrated various beneficial outcomes with 5azadC treatment including a reduction in left ventricular mass, improved cardiac contractility, decreased ROS production and reversed norepinephrine-induced changes in the expression of myotrophin and FHL2 (363). Other experimental non-nucleosidic small molecular inhibitors of DNA methylation such as RG108 (274) have been shown to improve contraction and relaxation in an *in vitro* engineered heart tissue model after afterload enhancement. The beneficial effect of these agents on aberrant cardiac remodeling in preclinical models has been demonstrated, yet the precise effect of these DNMT inhibitors on the different cell types of the heart involved in remodeling remains unknown.

Histone Modifications

Chromatin structure can play an important role in the regulation of gene expression and has been extensively studied. Chromatin is composed of a basic structure known as the nucleosome, ~145–147 bp of DNA wrapped around a wedge-shaped compact histone octamer composed of two copies each of the histone proteins H2A, H2B, H3 and H4 (398). The core histone proteins have amino -terminal tails which project out and make contact with adjacent nucleosomes and function as the principal sites for post-translational modifications (PTMs) (198). These are small chemical modifications to the amino acid side chains that are added and removed by a multitude of highly specific enzymes. These PTMs include acetylation, methylation, phosphorylation, poly-ADP-ribosylation, ubiquitination, deimination and sumoylation (22, 179). Modification at specific positions within histone tails can affect both the histone-DNA interactions and inter-nucleosomal interactions, facilitating remodeling of the chromatin structure and influencing the accessibility of transcriptional machinery binding to the DNA for active gene transcription (32). Two of the most well studied PTMs are histone acetylation/deacetylation and histone methylation/demethylation (**Figure 3**). Histone modifications are known to play a crucial role in cardiac development (211, 340) and therefore it is not surprising that alteration of these modifications contributes to pathological remodeling within the heart.

Histone Acetylation in Heart Failure

Histone acetylation is the process by which acetyl groups are added to ε-amino group of lysine side chains and is catalyzed by the histone aceytltransferase enzymes (HATs), using acetyl coenzyme A as a cofactor (22). There are 17 human HATs that are divided into five families based on sequence analysis. These include the GCN4-related N-acetyltransferase (GNAT) family (for example NAT6, GCN5 and HAT1), the CBP/p300 proteins, the MYST family (including MOF, MOZ, MORF, TIP60), $TAF_{II}250$ family (such as TAF1) and the steroid receptor coactivators (NCOA1, NCOA2, NCOA3) (209). Addition of acetyl groups results in neutralization of the charge interaction between the negatively charged DNA backbone and the positively charged lysine residues, promoting relaxed chromatin conformation (euchromatin) and active gene transcription. Histone acetylation of lysine 9 (H3K9ac) and lysine 14 (H3K14ac) are marks that correlate with gene activation (335). Acetylation marks are also associated with regulatory regions of the genome. Enhancer regions of the genome are distal regions that stimulate gene transcription and can now be notably identified by acetylation of lysine 27 on H3 (H3K27ac) (268). Removal of acetyl groups is carried out by histone deacetylase enzymes (HDACs) and results in chromatin condensation (heterochromatin) therefore repressing transcriptional competence (335). There are 18 HDAC members that are categorized into four classes (I-IV) based on their structure, substrate specificity, cellular location and tissue-specific expression. These include class I (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9, and 10), class III or sirtuins (SIRT1, 2, 3, 4, 5, 6, and 7) and class IV (HDAC 11) (75, 348).

The role of various HATs and HDACs in cardiac disease has been extensively studied. The use of preclinical models of heart failure that harbour genetic defects, either deletion or overexpression of HAT or HDAC enzymes, along with the use of small molecule inhibitors of their function have collectively demonstrated their involvement in how the heart responds to cardiac injury, including cardiac hypertrophy and fibrosis (**outlined in Table 3**), previously reviewed in (115, 352, 364).

One particular HAT that has been implicated in modulating heart failure is the acetyltransferase p300. Levels and activity of p300 within myocardial biopsies from patients with ischemic, dilated, or unspecified end-stage cardiomyopathy was significantly increased compared to that of non-failing control cardiac tissue (353). Deficiency of p300 in mice is embryonically lethal and can result in cardiac abnormalities such as reduced ventricular trabeculation and weaker contractions with abnormal expression of myosin heavy chain and alpha-actinin proteins (380).

Major emphasis has been placed on the role of HDACs in cardiac development and disease, focused mainly on class I and class II HDACs. HDAC involvement in cardiac pathology is complex as different HDACs; even from the same class can have a differential influence on how the heart responds to cardiac injury through either promotion or attenuation of cardiac remodeling (115). Levels of class I HDACs, HDAC1 and HDAC2, have previously been shown to be increased in both infarcted and non-infarcted myocardium of chronic heart failure (245). Mice with global knockout for HDAC2 that received catecholamine-induced cardiac damage did not develop cardiac fibrosis, re-expression of fetal cardiac genes (Nppa, Myh7 and Acta1) or cardiac hypertrophy (330). HDAC2 deficient mice were also protected from hypertrophy in response to transaortic constriction (TAC)-induced pressure overload, suggesting that HDAC2 plays a role by promoting aberrant cardiac remodeling (330).

Class II HDACs, HDAC5 and HDAC9 demonstrate a cardiac protective role both in normal hearts and in response to cardiac injury. Evidently, mice deficient in HDAC5 (53) and HDAC9 (386) were found to have age-dependent cardiac hypertrophy even in the absence of any cardiac injury. In the setting of pressure overload, these null mice displayed an exaggerated hypertrophic response associated with the ability of these HDACs to interact with and repress transcription of MEF2 target genes involved in driving cardiac hypertrophy, such as Nppa and Nppb (53, 386). Interestingly, double knockout of HDAC5 and HDAC9 resulted in severe embryonic cardiac abnormalities including ventricular septal defects and thin ventricular walls (53). Polymorphisms in HDAC9 have also been linked to increased risk and severity of coronary artery disease (30, 125, 344). Another member of the class II HDAC family, HDAC6, has previously been shown to contribute to cardiac systolic dysfunction through effects on sarcomeric proteins and impairing contractility of myofibrils (80). In response to AngII infusion, HDAC6 KO mice developed similar levels of aberrant cardiac remodeling to wild type mice (fibrosis and hypertrophy) but retained preserved ejection fraction with increased myofibril force generation (80). This indicates that HDAC6, while not involved with remodeling of the heart, can still contribute to cardiac dysfunction through impairing cardiac contractility.

Class III HDACs or sirtuins are characterized by their requirement of nicotinamide adenine dinucleotide (NAD+) for their enzyme activity (7, 144, 331) and have been implicated in the pathogenesis of cardiovascular disease and heart failure previously reviewed in (213) (**Table 4**).

Pharmacological Targeting of Histone Acetylation and Deacetylation

Involvement of histone acetylation in aberrant cardiac remodeling has been demonstrated by numerous studies that portray the therapeutic benefit of pharmacological inhibition of HAT and HDAC activity in preclinical models of heart failure. Curcumin or diferuloylmethane, a polyphenol that acts to inhibit p300 HAT activity, has been shown to attenuate cardiac remodeling in rat models of hypertensive heart disease and MI (225, 226). Inhibitors of HDAC activity (HDACi) are categorized into four groups; hydroxamates (Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA)), benzamides (Entinostat), cyclic peptides (depsipeptide, also known as romidespin) and aliphatic acids (e.g. Valproic acid) (365). They can also have both broad inhibitory actions

and inhibit several classes of HDACs, such as SAHA and TSA, whereas others can selectively inhibit specific classes of HDACs such as apicidin, depsipeptide and SK7041 which are selective class I HDACis (236, 365) and specific for HDAC enzymes such as MPT0E014, a selective inhibitor of HDAC1, 2 and 6 (182). HDAC inhibitors have demonstrated therapeutic benefit in numerous preclinical models of heart failure (352), showing a reduction in hypertrophy (106, 164, 177, 248), fibrosis (148, 160) and inflammation (1, 216) resulting in improvements in cardiac function. Recent data investigating the role of HDAC3 demonstrated that a selective inhibitor, RGFP966 prevented diabetic cardiomyopathy in a preclinical model of diabetes, with treated animals displaying a sustained reduction in inflammation, oxidative stress and cardiac fibrosis (369). This work indicated that HDAC3 inhibition enhanced acetylation at the promoter region of the nuclear phosphatase DUSP5 gene resulting in its increased expression and ability to block activation of ERK1/2 activity (369).While three HDAC inhibitors have been FDA-approved (SAHA, romidespin and belinostat) for the treatment of cancers, such as T-cell lymphoma (228), none have yet received approval for the treatment of heart failure. Activators of class III HDAC/Sirt activity, such as resveratrol, have also demonstrated beneficial effects on aberrant cardiac remodeling which seem to be independent of reducing cardiac injury. SHR rats demonstrated a significant reduction in cardiac hypertrophy with improved cardiac function when treated with 2.5mg/kg/day for 10 weeks (328). The beneficial effects of resveratrol have been demonstrated in other pre-clinical models of heart failure (272, 323) and are thought to be due to reducing oxidative stress and preserving mitochondrial function.

Histone Methylation and its Role in Heart Failure

The process and consequences of histone methylation are more complex than acetylation. Methylation can occur on both lysine and arginine residues; residues can be methylated to different degrees (mono-, di- or tri-methyl for lysines and mono- or di-methyl for arginine) and depending on the residues that are methylated and the degree of methylation, gene transcription can either be repressed or augmented (178, 179, 290). Currently it is known that histone methylation is a dynamic process. Addition of methyl groups is carried out by methyltransferase enzymes, including the SET-domain-containing family and the disruptor of telomeric silencing 1-like (DOT1L)/KMT4 family. Previously, methylation of histones was considered a relatively stable epigenetic mark however the discovery of histone demethylase enzymes, which catalyse the removal of the methyl groups, demonstrated that histone methylation is a highly dynamic modification (300). The two main families of demethylase enzymes are the LSD demethylases (LSD1/KDM1A and LSD2/KDM1B) and the Jumonji C-domain containing demethylases (JMJDs) (34, 300). Methylation at specific lysine residues on histone protein 3, H3K4, H3K26 and H3K79 are associated with active gene transcription whereas methylation at H3K27, H3K9 and H3K20 are associated with transcriptionally silenced heterochromatin (178). Tri-methylation of H3K36 (H3K36me3) is normally found in the gene bodies of actively transcribed genes, however it also is associated with transcriptional repression (338). Protein complexes that also act to modify chromatin structure through regulation of histone methylation include the Trithorax Group (TrxG) and the polycomb group (PcG) complexes (291). TrxG complex promotes gene transcription through their H3K4 trimethylase activity, whereas PcG complexes have H3K27-specific trimethylase activity (such as Enhancer of zeste homolog 2 (EZH2) activity in the PcG2 complex), silencing gene transcription (291). Both of these complexes have been shown to be critical for normal cardiac development and function (343).

Alterations in histone methylation have been previously documented in failing human and rodent hearts (**Table 5**) (9, 137, 159, 334, 388). Using chromatin immunoprecipitation (ChIP) coupled to pyrosequencing, Kaneda and colleagues have demonstrated marked differences in tri-methylation for both K4 and K9 residues of histone H3 in both human heart failure and Dahl salt-sensitive rat cardiac tissue (159). Their analysis revealed enrichment of genes involved in signalling pathways related to cardiac function, such as calcium signalling, synaptic long-term regulation, G protein coupled and nitric oxide signalling, located close to these methylation marks, especially H3K4me3 (159). The PAX interacting (with transcription-activation domain) protein 1 (PTIP)-associated HMT complex, which contains PTIP, cofactors Ash2L, RbBP5, WDR5, the

KDM6A lysine demethylase and the SET domain containing methyltransferases KMT2B and KMT2C, are important in regulating H3K4me3 marks at actively expressed genes (253). Studies have demonstrated the importance of these complexes in cardiac structure and function as reduction in H3K4me3 marks in the heart can result in aberrant calcium handling and ventricular arrhythmias upon β-adrenergic stimulation (309) while promoting maladaptive cardiac remodeling in pressure overload (308).

Changes in methylation of histones have previously been implicated with re-expression of fetal genes in the failing heart (137). Decreased H3K9 methylation and binding of Heterochromatin protein 1 in the promoter regions of Nppa and Nppb was found to be associated with their increased expression in failing human myocardium (137). Upregulation of JMJDs (JMJD1A, JMJD2A, and JMJD2B) was also apparent in this study, with expression of JMJD1A positively correlating with reactivation of fetal genes, Nppa and Nppb (137). Interestingly, this study also noted that recruitment of JMJD2A to the promoter region of Nppa was only found to be increased in ischemic cardiomyopathy and not in dilated cardiomyopathy (137). Levels of JMJD2A have also been found to be augmented in patients with hypertrophic cardiomyopathy, suggesting that both levels and recruitment of demethylase enzymes can be altered in specific cardiac pathologies (388).

Repression of Myh6 and Atp2a2 gene expression are changes established in the failing myocardium (137). A study by Han and colleagues has highlighted the potential role of histone methylation silencing of Myh6 (334). In a small cohort of human hypertrophic hearts linked to chronic hypertension and/or morbid obesity, changes in expression were mirrored by an enrichment of H3K9me2 in the Myh6 promoter region along with increased expression of G9a histone methyltransferase (334). Other mechanisms could be due to lower levels of active methylation marks in its gene promoter. Anagrisano *et al*., evaluated chromatin marks in the promoter regions of ATP2A2 and Myh7 genes, which were found to be aberrantly expressed in the hearts of mice subjected to chronic pressure overload (9). Decreased transcriptional activity of ATP2A2 was associated with reduced H3K4me2 and increased repressive H3K9me2, H3K27me3, H3K36me2 marks with the opposite being found for Myh7, promoting its expression(9). The increased methylation of H3K36 was associated with a reduction in lysine-specific demethylase KDM2A at the ATP2A2 promoter (9).

The abnormal gene expression demonstrated in both clinical and experimental models of heart failure, as a result of aberrant changes in histone methylation and demethylation, highlight the important role that this epigenetic process plays in the pathogenesis of cardiac disease. Further insight into the cellular identity in which these changes take place could facilitate the development of novel targeted interventions to reverse these pathological changes and improve clinical outcome.

Epigenetic Regulation of Cardiomyocyte Function

Whilst cardiomyocytes are arguably the most important functional cell type in the heart they only account for 20-30% of the total cell population (277). Cardiomyocytes are non-dividing, terminally differentiated cells that respond to pathological stress and injury by undergoing hypertrophy. Due to their non-proliferative nature, and the assumption that new epigenetic modifications are primarily introduced into dividing cells, the influence of epigenetic modifications on regulating cardiomyocyte phenotype was generally overlooked. *Ex vivo* investigation into the role of epigenetic regulation of myocyte function in both healthy and disease contexts has been limited on account of their terminally differentiated state and the fact that genomic DNA isolated from whole heart tissue contains such a variety of contributing cell types. Recent evidence using various experimental animal models and novel methods to isolate cardiomyocyte nuclei from cardiac tissue (31, 109) is building a case for the idea that dynamic changes in epigenetic modifications plays a more significant role in regulating cellular function and response of the myocyte than previously thought.

DNA Methylation in Cardiomyocytes

DNA methylation in myocytes was initially considered redundant due to their terminally differentiated state. However, with *de novo* methylation (DNMT3A and DNMT3B) and demethylation (TET enzymes) which can alter methylation status and gene expression, it is now considered that DNA methylation can have the potential to modulate gene expression in postnatal cardiomyocytes (**Table 6**). In order to investigate changes in DNA methylation, cardiomyocyte nuclei can now be isolated and purified from both rodent and human cardiac tissue by flow cytometry (fluorescent activated cell sorting) or magnetic-assisted nuclei sorting using an antibody against pericentriolar material 1 (PCM1) (31, 264). PCM1 has been validated as a marker of cardiomyocyte nuclei in transgenic mice with cardiomyocyte-specific expression of histone H2B-mCherry (109).

Dynamic changes in cardiomyocytes have been shown to occur during the postnatal period. A study by Gilsbach *et al*., compared methylation profiles of cardiomyocytes from new-born and adult hearts (109). They found that 121 genes gained and 313 lost methylation in the gene bodies during the postnatal period suggesting the role of *de novo* methylation. The authors reported that genes hypermethylated postnatally were involved in muscle contraction, cardiac morphogenesis, cell differentiation and many other biological processes. Investigating changes in DNA methylation that may occur in the functional adaptation of the heart during normal development, such as isoform switching in the expression of sarcomere proteins, the authors demonstrated the change from the fetal troponin I (*Tnni1)* to the adult troponin 3 (*Tnni3)* was accompanied by *de novo* methylation of *Tnni1* and demethylation of Tnni3 with partial expression of *Tnni1* being revived in adult cardiomyocytes. While methylation changes in cardiomyocytes subjected to 3 weeks of pressure overload, were modest compared to those that were seen during myocyte maturation, at disease-associated differential methylation regions the methylation profile partially resembled that of fetal cardiomyocytes (109).

To investigate the involvement of DNMT3 enzymes in cardiomyocytes and whether *de novo* methylation plays a role in altering cardiomyocyte response to injury, various groups have started to employ cardiac-specific DNMT knock out (KO) models, in particular with the DNMT3 isoforms. The crucial involvement of these enzymes in organism development has made creation of gene KO models to be very difficult. DNMT3B homozygous KO mice die during gestation with severe developmental defects (246) and DNMT3A homozygous knockout mice appear normal at birth but become runted and die at about 4 weeks of age (246). Cre-lox recombination has enabled the development of inducible DNMT3 isoform KO mice using cardiac specific promoters such as myosin heavy chain 6 (*Myh6*), troponin T (*Tnnt2*) and atrial myosin light chain (*Myl7*) (82). Cardiomyocyte-specific deletion of DNMT3B in 8 week old mice resulted in blunted hypertrophic responses accompanied with chamber dilation and decreased cardiac function when subjected to TAC and isoprotenerol (337). Interestingly, mice that had sham surgery but had sustained loss of DNMT3B showed evidence of reduced contractility and chamber dilation resulting from widespread interstitial fibrosis and myo-sarcomeric alterations. Differential methylation of three CpGs within intron 27 resulted in alternative splicing of *Myh7* in mice with DNMT3B deletion subjected to pressure overload (337).

Other groups have generated inducible DNMT3A/DNMT3B double KO mice, where cardiomyocytes lack the catalytic domains of DNMT3A and DNMT3B, to investigate whether *de novo* DNMTs in cardiomyocytes play a role in the response to pressure overload (243). DNMT3A/DNMT3B deletion had no effect on cardiac hypertrophy as double KO mice still had significantly increased LV mass, increased LV diameter, and interstitial fibrosis along with decreased ejection fraction and increased lung weight (243). They carried out genome wide assessment of gene expression in ventricular tissue using an Illumina bead ChIP array to determine any genes that where differentially expressed in DNMT3A/DNMT3B KO mice, both with and without pressure overload. In response to pressure overload they found 17 genes that showed differential expression as a result of genotype rather than disease state. Three genes (*Aldh1l1, Krt8 and Sclc9a3*) were selected for pyrosequencing analysis based on their increased expression in the KO model, which was enhanced in response to pressure overload. Hypomethylation at CpG sites in promoter regions of these genes was found in cardiomyocytes

isolated from KO mice under sham conditions. Expression of these genes was negatively correlated with methylation at these CpG sites in sham conditions with expression further increased in response to pressure overload (243).

It is interesting to see differences in cardiac phenotypes found between the two different DNMT cardiomyocyte KO models in response to TAC. While differences were noted between these models including KO of one DNMT compared to a double KO of two DNMT3 (3A and 3B) active isoforms along with differential timing of gene knockdown, the major finding from these studies is that *de novo* methylation is an active process in postnatal cardiomyocytes and can influence gene expression in these cells. Recent work carried out by Fang *et al*., has highlighted the importance of DNMT3A in regulating cardiomyocyte gene expression, structure and function (90). siRNA mediated downregulation of DNMT3A but not DNMT3B in embryonic cardiomyocytes resulted in abnormal structural and physiological properties including disruption in sarcomeric Z band organisation, impaired calcium signalling, decreased beat rate variability and loss of both contractility and contractile force (90). These structural and functional myocyte defects were associated with differential gene expression in pathways involved in calcium signalling, endothelin-1, RAAS, β-adrenergic and insulin receptor signalling (90). While Fang and colleagues have highlighted the importance of DNMT3A in developing cardiomyocytes, the other studies described above suggest that mice with cardiomyocyte-specific DNMT3A deletion mount a normal hypertrophic response to pressure overload. Further work is therefore needed to confirm whether DNMT3A is implicated in post-natal cardiomyocyte dysfunction after cardiac injury.

While DNMT1 was generally considered to be redundant in cardiomyocytes because of their post-mitotic nature, it is becoming increasingly apparent that DNMT1 levels can be altered in response to cardiac injury, especially in states of metabolic stress. DNMT1 expression was found to be elevated in cardiomyocytes exposed to high levels of glucose (57), homocysteine (55) and most recently an environmental pollutant, phenanthrene (142). In these cells, the increased DNMT1 levels were accompanied by cellular dysfunction such as increased oxidative stress and myocyte hypertrophy (55, 142). The potential role of DNA methylation in cardiomyocyte hypertrophy is further supported by evidence that pharmacological inhibition of DNMT activity in spontaneously hypertensive rats attenuates hypertension-induced increases in myocyte cross-sectional area (351). Interestingly, the microRNA, miR-133a, known to be involved in cardiac hypertrophy (48), was determined as an important regulator of DNMT1 expression. Antagonizing the effects of miR-133a promoted expression of DNMT1 in cultured cardiomyocytes whereas overexpression resulted in transcriptional silencing (57). Collectively these studies highlight that aberrant regulation of the maintenance methylating enzyme DNMT1 by non-coding RNAs in post-mitotic cells such as cardiomyocytes may be involved in promoting cellular dysfunction in response to injury.

The relevance of hydroxymethylation of DNA by TET enzymes has recently been implicated as a key cellular process that is involved in regulation of cardiomyocyte gene expression and cellular function in response to pressure overload (116). Changes in hydroxymethylation distribution occur from the transition from enrichment of 5hMeC at intergenic regions in fetal cells to enrichment on the gene bodies of adult cardiomyocytes. In the situation of pressure overload induced by TAC, the distribution of 5hMeC reverts back to that of the fetal pattern. Reversion back to this fetal hydroxymethylation pattern, was found to be associated with upregulation of cardio-specific genes involved in extracellular matrix and actin skeleton organization and repression of genes related to energy metabolism including those involved in the tricarboxylic acid cycle and fatty acid oxidation (116). In terms of 5MeC oxidation, TET2 was found to be the most abundantly expressed member of the TET family in cardiomyocytes and interestingly knockdown of TET2 led to alterations in 5hMeC at specific loci rather than global hydroxymethylation alterations. The authors found that fetal genes were regulated by hydroxymethylation of DNA in hypertrophied cardiomyocytes, with genes such as *Myh7* being regulated by genic and enhancer hydroxymethylation. Reactivation of gene expression was associated with hydroxymethylation at intergenic enhancer regions whereas inhibition of TET2 function by gene knockdown resulted in decreased hydroxymethylation at intragenic regions leading to in decreased expression of *Myh7* (116).

Histone Acetylation in Cardiomyocytes

Numerous studies have implicated the ability of histone acetylation and deacetylation to influence the hypertrophic phenotype in cardiomyocytes (**Table 7**). The involvement of HATs in promoting hypertrophy in cardiomyocytes was demonstrated using a model of catecholamine-induced stress in primary cardiac myocytes in culture (120, 374). Over expression of p300 or CBP was shown to induce myocyte hypertrophy *in vitro*, with acetylation activity of these enzymes being increased in response to phenylephrine (120). This phenylephrine-increased activity of p300 in myocytes has been shown to augment the DNA binding capacity of GATA4, a pro-hypertrophic transcription factor involved in driving increased cardiomyocyte size and sarcomere organization (5). Acetylation of the GATA4 transcription factor by p300 was also shown to increase myocyte cross-sectional diameter in response to ischemic injury (219). Other cardiomyocyte-specific transcription factors are also regulated by p300. In response to pressure overload, cardiac-specific over-expression of p300 was found to be associated with increased *de novo* acetylation of myocyte enhancer factor-2 (Mef2) with enrichment for transcripts in p300-regulated promoters, promoting cardiac hypertrophy and dysfunction (353). This ability of p300 to induce hypertrophy of cardiomyocytes can be prevented by pharmacological inhibition of p300 activity (3, 226).

HDAC enzymes are known to regulate myocyte growth responses to cardiac stress. Studies looking at the role of HDAC2 in development and injury have yielded interesting findings (222, 330). Over-expression of HDAC2 under control of the Myh6 promoter resulted in significant myocyte hypertrophy. One potential regulatory mechanism of HDAC2 to induce hypertrophy is through the PI3K-Akt-Gsk3beta pathway. Over-expression of HDAC2 resulted in decreased expression of Inpp5f, increasing phosphorylation and inactivity of Akt, Pdk1 and Gsk3beta (330). Further gain- and loss-of-function investigations into the role of Inpp5f indicated that it plays a particularly important role in driving myocyte hypertrophy in response to cardiac stress (393). Myocytes deficient in Inpp5f did not experience any cardiac abnormalities under basal conditions; however, in the presence of adrenergic stimulation, displayed a significant hypertrophic response compared to wild type controls (393). HDAC2 may also modulate other regulatory factors driving myocyte hypertrophy. A study by Kee and Kook, using promoter mapping analyses of the Nppa promoter found that expression and binding of Krüppel-like factor 4 (KLF4) was significantly reduced upon HDAC2 activation by hypertrophic agonists (phenylephrine or partial aortic constriction) (163). Furthermore, *in vitro* knockdown of KLF4 resulted in increased expression of Nppa, protein synthesis and stress fibre formation (163). Interestingly, cardiac-specific loss of HDAC2 or HDAC1 had no impact on isoproterenol and pressure overload-induced hypertrophic responses (222). Double knockout of both genes resulted in major cardiac defects such as cardiac arrhythmias and dilated cardiomyopathy, leading to death during the postnatal period (222) and was associated with altered expression of genes encoding myofibrillar proteins (Tnni1, Tnni2) and specific calcium channel subunits (CACNA1H, CACNA2D2).

Other class I HDACs have also been implicated in regulating cardiomyocyte growth. Cardiac-specific deletion of HDAC3 resulted in apparent cardiac hypertrophy at 4 weeks of age and this was dramatically increased at 12 weeks of age with alterations in myofibril structure and mitochondrial structure (223). Increases in expression of Nppa, Nppb and Acta1 were also seen in myocyte-specific HDAC3 KO mice (223). The study concluded that the cardiac hypertrophy was associated with metabolic dysfunction with myocardial lipid accumulation and elevated triglyceride levels that were attributed to excessive activity of the peroxisome proliferator activated receptor (PPAR) α receptor (223). Interestingly, transgenic overexpression of HDAC3 in cardiomyocytes did not show spontaneous cardiac hypertrophy or increased sensitivity to isoproterenol but did however result in increased myocyte proliferation at birth with decreased expression of negative regulators of cell cycle progression (329). The involvement of class II HDACs in driving cardiomyocyte hypertrophy has been extensively reviewed previously (352).

Pharmacological inhibition of HDAC enzymes has been shown to attenuate the pathological hypertrophic phenotype in cardiomyocytes. Antos *et al*. assessed the hypertrophic response of primary cardiomyocytes when treated with HDACis (including TSA). They found that HDACi

treatment resulted in blunted hypertrophic responses, showing reduced protein synthesis and inhibition of fetal gene re-expression (10). It has also been demonstrated that HDAC inhibition may provide a cardio-protective effect to myocytes/myoblasts in the case of ischemic injury (387). One potential mechanism by which the beneficial effect of TSA could be mediated is by activation of ubiquitination-dependent proteasomal degradation of HDAC4 through sumoylation (83). Collectively, these results are recapitulated in the numerous studies that have previously demonstrated anti-hypertrophic and cardio-beneficial effects of pharmacological inhibition of HDAC activity. Evidence is also emerging to implicate the involvement of long non-coding RNAs (lncRNA) in the development of heart disease and cardiac dysfunction via regulation of histone modifications. One potential novel mechanism by which HDACis can reduce cardiac hypertrophy is through modulation of lncRNA expression (124). A cluster of alternatively spliced transcripts of Myh7, known as Mhrts, have been shown to have cardio-protective effects through interaction with the helicase domain of Brg1 complex, which is known to induce pathological switch of Myh6/7 gene expression in response to stress (124). Under myocardial stress situations, such as pressure overload, it was shown that the Brg1-HDAC-PARP chromatin repressor complex inhibits the transcription of Mhrt, promoting cardiomyocyte hypertrophy (124). Use of the HDACi, TSA, restored expression of Mhrt, preventing Brg1 from recognizing its chromatin targets (124).

Sirtuins (Class III HDACs) have also been implicated in modulating the hypertrophic cardiomyocyte phenotype. Cardiomyocyte-specific deletion of SIRT6 in adult mouse hearts resulted in significant cardiac hypertrophy, measured by echocardiography and histology. The mechanism by which Sirt6 appears to act as a negative regulator of cardiac hypertrophy is through both deacetylating H3K9 and negatively regulating the expression of Insulin-like growth factor (IGF) signalling–related genes by suppressing the transcriptional activity of c-Jun (316). Other Sirtuins have also been implicated in regulating cardiomyocyte hypertrophy including Sirt1 (314) and Sirt3 (315). Altogether, these studies highlight the potential of acetylation and deacetylation in regulating the hypertrophic phenotype in cardiomyocytes in response to cardiac injury.

Histone Methylation in Cardiomyocytes

The process of histone methylation is crucial for normal cardiomyocyte development, homeostasis, and response to injury (**Table 8)**. This was demonstrated by Wamstad and colleagues when they induced cardiomyocyte differentiation from embryonic stem cells in a stepwise manner and found dynamic changes in histone modifications, H3K4me1, H3K4me3 and H3K27me3 that govern cardiomyocyte differentiation (340). Methyltransferase enzymes such as DOT1L have been demonstrated to mediate H3K79me2 modifications, which are critical for regulation of gene expression during cardiomyocyte differentiation and maturation (252). Further importance of these modifications is that *de novo* mutations in genes involved in production, removal or reading of H3K4me and H3K27me contribute to the development of severe congenital heart disease (384).

Alterations in global histone methylation have been associated with hypertrophic response in *ex vivo* isolated cardiomyocytes (252). Cardiomyocytes isolated from the hearts of mice that experienced one week of TAC-induced pressure overload demonstrated altered epigenetic profiles at enhancer and promoter regions with dynamic and locus-specific alterations in both active (H3K4me3 and H3K79me2) and repressive (H3K9me2, H3K9me3, and H3K27me3) histone marks (252). These histone marks in the promoter regions correlated with expression of a large group of genes in cardiomyocytes that were differentially expressed (325 genes out of 1109) as a result of pressure overload (252).

Activation of gene expression in response to cardiac injury in cardiomyocytes is essential to maintain cardiac integrity. Using a cardiomyocyte-specific KO model for PTIP-associated HMT complex, Stein and colleagues demonstrated that different cardiac phenotypes occur in response to different cardiac insults, with H3K4me3 methylation being an important regulator (308, 309). PTIP KO mice that experienced β-adrenergic stimulation by isoproterenol and caffeine developed ventricular arrhythmias and abnormal calcium handling, but did not experience cardiac

hypertrophy (309). Interestingly, however, in TAC-induced pressure overload, PTIP KO mice experience maladaptive cardiac remodeling with significant cardiac dilation and impaired cardiac function. These aberrant changes were found to be accompanied by altered gene expression of ADRA1A, ADRA1B, JUN, ATP2A2, ATP1A2, SCN4B, and CACNA1G suggesting that precise regulation of these genes in cardiomyocytes is important for proper adaptive responses to injury.

The role of histone demethylation in the hypertrophic response of cardiomyocytes has been clearly demonstrated by Zhang and colleagues (388). In the absence of injury, mice either lacking or over-expressing JMJD2A in cardiomyocytes showed normal cardiac morphology and function (388). However, in response to pressure overload, overexpression of JMJD2A conferred an exacerbated hypertrophic response and the opposite was seen with deletion of JMJD2A in cardiomyocytes, which attenuated hypertrophy with improved cardiac function (388). The authors found that JMJD2A promotes myocyte hypertrophy through binding to the promoter of four-and-a-half LIM domains protein 1 (FHL1), increasing its expression via transcription factors such as serum-response factor and myocardin (388). Interestingly other JMJD enzymes altered in human heart failure, such as JMJD1A have been shown *in vitro* to compensate for knockdown of JMJD2A in neonatal cardiomyocytes. Knockdown of either JMJD1A or JMJD2A alone did not impact on the regulation of fetal gene expression (Nppa and Nppb) (137). Double knockdown of both JMD1A and JMJD2A in neonatal cardiomyocytes resulted in increased H3K9me2 and H3K9me3 at the promoter regions of Nppa and Nppb, resulting in a moderate decrease in their expression (137).

As expected the involvement of histone methylation in regulating gene expression in the failing heart is complex and involves orchestration and collaboration of other cellular processes that regulate gene transcription, including other epigenetic processes such as DNA methylation and non-coding RNAs, to drive a pathological hypertrophic myocyte phenotype. Han *et al*, have demonstrated that transcriptional silencing of Myh6 in response to pressure overload is achieved in a step by step manner whereby the ATP-dependent chromatin remodeling complex, Brg1, recruit histone methyltransferases (G9a/Glp) which then recruit DNMT3A, leading to DNA hypermethylation and stable Myh6 repression (334). The role of histone methylation in this cascade was demonstrated by doxycycline-inducible deletion of G9a in adult cardiomyocytes. G9a-deficient mice subjected to pressure overload displayed only mild hypertrophy at two weeks and went on to demonstrate significant improvement in both hypertrophy and cardiac function at 8 weeks compared to littermate controls (334). With these improvements, TAC-induced H3K9me2 of Myh6 was significantly reduced as was binding of DNMT3A to the Myh6 promoter and CpG methylation (334). LncRNAs have also been demonstrated to interact with chromatin remodeling complexes and modulate histone modifications for induction of cardiac hypertrophy in myocytes. A recent study published by Wang *et al.,* has highlighted the role a lncRNA known as cardiac hypertrophy associated epigenetic reader (Chaer), which is located predominantly in the nucleus, expressed in cardiomycoytes and regulates the development of myocyte hypertrophy in response to cardiac injury (349). Chaer-deficient mice displayed no functional or morphological differences in cardiac phenotype under basal conditions, and showed attenuation of pathological remodeling in response to TAC (349). Suppression of phenylephrine-induced hypertrophy and expression of hypertrophic genes (Nppa, Myh7 and Acta1) was achieved after knockdown of Chaer in ventricular myocytes. Overexpression of Chaer, even in the absence of beta-adrenergic stimulation resulted in enlargement of myocytes and increased expression of Myh7 and Acta1. The mechanism by which Chaer promotes hypertrophy is through its interaction with the PcG2, mediated by mTOR signalling, preventing it from binding to and carrying out H3K27 methylation at the promoters of hypertrophic genes (349). These findings were demonstrated in both human and rodent cardiomyocytes suggesting a highly conserved mechanism of stress induced regulation of myocyte hypertrophy (349).

Epigenetic Regulation of Fibroblast Function

Fibroblasts are spindle shaped mesenchymal cells with oval nuclei that play a functional role in the development and maintenance of the structural and physiological integrity of organ systems in

the body. While fibroblasts are found all over the body and are primarily involved in the production and turnover of ECM, in the heart these cells can also influence important physiological aspects including angiogenesis, contractility and cardiac electrophysiology (44, 262, 306).Throughout the literature fibroblasts are usually identified by various cellular markers including surface markers (Discoidin domain receptor 2 (DDR2), Fibroblast surface antigen and Fibroblast activation protein 1 (FAP-1)), intracellular markers (αSMA, vimentin and Fibroblast-specific protein 1 (FSP-1)) and secreted products (CTGF/CCN2 and Collagen I) which generally have been shown to be highly expressed by fibroblasts in the diseased heart (105). Unfortunately, specificity of these markers to cardiac fibroblasts remains an issue as they are also expressed by other cell types including endothelial cells, smooth muscle cells, lymphocytes and other mesenchymal cells including pericytes, as previously summarised by Ma and colleagues (199). Initially, fibroblasts were considered to account for 60-70% of the total population of cells within the heart, however recently this figure has been revised with evidence suggesting that fibroblasts account for <20% of the total non-myocyte cardiac cell population (258). None the less, fibroblasts are still a cell of great interest due to the role they play in wound healing and cardiac pathology.

The functional abilities of cardiac fibroblasts are mediated through transdifferentiation into cardiac myofibroblasts that have high ECM synthetic abilities. The exact origin of myofibroblasts in the heart is controversial, with various sources being hypothesized under stressful conditions including proliferation of resident cells (224), recruitment of bone marrow-derived progenitor cells (332), epithelial-to-mesenchymal transition (EMT) of epicardial cells (43) and endothelial-to-mesenchymal transition (EndMT) of endothelial cells (4). Regardless of the precise source of these cells, it is undisputed that, injurious stimuli including ischemia, reperfusion and mechanical stretch activate this phenotypic transition with fibroblasts becoming hyper-activated and adopting a pro-fibrotic myofibroblast phenotype. Unlike in organs such as the skin, where the myofibroblasts are transient and undergo apoptosis after resolution of the normal wound healing processes (81), myofibroblasts have been shown to persist in the heart, months and even years after the initial cardiac injury (157, 357). Evidently, *ex vivo* culture of fibroblasts from fibrotic environments demonstrates that these cells maintain their cellular characteristics and proliferative capacity (156).These cells are also known to become sensitised and can further release pro-inflammatory mediators such as cytokines and chemokines which subsequently enhances pathological remodeling in the injured heart (262).

Whether in the case of reparative fibrosis in myocardial infarction, reactive fibrosis in pressure overload or aging, the exact driving force behind this persistent hyperactive myofibroblast remains unknown. What is known is that acquisition of the activated myofibroblast phenotype is accompanied by changes in gene expression (256). Epigenetic mechanisms such as DNA methylation and histone modifications could therefore underpin this aberrant persistent cellular phenotype that ultimately contributes to the development and progression of cardiac failure.

DNA Methylation in Fibroblasts

Regulation of fibroblast phenotype and function by DNA methylation (**Table 9**) has been widely studied in numerous fibrotic conditions such as renal fibrosis, hepatic fibrosis, pulmonary fibrosis, cardiac fibrosis and systemic sclerosis.

Within the heart several genes have been identified whereby methylation of their promoter regions modulates their expression and impacts fibroblast/myofibroblast function. Xu *et al.* initially demonstrated that hypermethylation of the RAS Protein Activator Like 1 gene (RASAL1), which functions to inhibit Ras-GTP activity, along with its transcriptional silencing was found in both a mouse model of pressure overload and in human fibrotic heart tissue from patients with end-stage heart failure (366). They found that this aberrant promoter methylation of RASAL1 promoted EndMT of human coronary endothelial cells (366), can be induced by hypoxia and is mediated by DNMT3A (367). Hypermethylation and transcriptional silencing of RASAL1 has been implicated in fibroblast activation and fibrogenesis in numerous fibrotic pathologies including hepatic stellate cells (HStCs) in hepatic fibrosis (206, 324), renal fibroblasts in renal fibrosis (27)

and human trabecular meshwork cells in glaucoma (215). Silencing of RASAL1 expression through promoter hypermethylation is thought to be mediated through MeCP2(324). Another gene that has been implicated in aberrant fibroblast activation and proliferation is Ras-association domain family 1 isoform A (RASSF1A), which functions as a tumour suppressor gene and is involved in the negative regulation of biological processes such as cell proliferation and cell survival (13). RASSF1A has an important role in the heart as systemic knock out of the gene results in an exaggerated fibrotic response after pressure overload (79). Interestingly, cell specific expression of this gene in the heart can have differential functional consequences, with loss of RASSF1A expression in cardiac fibroblasts advocating increased fibroblast proliferation and survival (79). DNA methylation seems to implicated in the regulation of RASSF1A expression in fibroblasts, as PDGF-activated fibroblasts demonstrate increased levels of DNMT3A in combination with reduced levels of RASSF1A (325). siRNA knockdown of DNMT3A restored RASSF1A expression and inhibited the pro-fibrotic phenotype (325). Other examples of genes being epigenetically silenced by DNA methylation include the collagen suppressor gene FLI1 in dermal fibroblasts (346), PTCH1 in hepatic myofibroblasts (377)and Pck2 in pulmonary fibroblasts (377).

With all fibrotic conditions, hypoxia is a unifying factor that is now appreciated to modulate fibroblast gene expression through DNA methylation. Watson *et al.,* have shown that hypoxia (1% oxygen) for 8 days induced global DNA hypermethylation in the genomes of cardiac fibroblasts. DNA hypermethylation was mirrored with increased expression of DNMT enzymes (DNMT1 and 3B) along with fibrotic markers (collagen 1 and αSMA) (350). Increased global DNA methylation induced by hypoxia has also been demonstrated in human pulmonary fibroblasts (273). On a gene specific manner, hypoxia-induced reduction of Thy-1 expression in these fibroblasts was associated with hemi-methylation of its gene promoter. Methylation of the Thy-1 promoter in fibroblasts can also be induced by *in vitro* treatment with pro-fibrotic cytokines such as TGFβ1, which acts to increase DNMT activity (241). Thy-1 deficient fibroblasts have previously been shown to be more resistant to apoptosis and have a fibrogenic phenotype (283).

Most studies that find aberrant gene expression as a consequence of DNA methylation have demonstrated reversal by pharmacological inhibition of DNMT activity or through intrinsic demethylation processes. The main DNMT inhibitor used in these investigations to reverse the hyperactive fibroblast phenotype is 5-azadC, whereby treatment of activated fibroblasts resulted in a reduction of the main fibrotic markers such as αSMA, collagen 1 or collagen 3 or a reduction in cell proliferation (215, 273, 324, 346, 350, 377). Inhibition of DNMT activity by 5aza has also demonstrated anti-fibrotic action on activated fibroblasts. In the context of the heart, inhibition of DNA methylation with 5aza resulted in a significant decrease in expression of collagen 1 and 3 in myofibroblasts derived from human ventricular cardiac fibroblasts (351). Exposure of fibrotic human kidney and pulmonary fibroblasts to 5aza displayed similar *in vitro* results (27, 141). Of note, attenuation of the fibrotic phenotype by 5aza in pulmonary fibroblasts was attributed to a reduction of bone morphogeneic protein (BMP)-endothelial cell precursor-derived regulator (BMPER) expression, which modulates TGFβ/BMP signalling by interacting directly with BMP's (BMP-2, -4 and -6)(141). BMPs are growth factors which exert diverse effects on target tissues with increased and decreased BMP signalling activity present in many cardiovascular diseases (227). Reduced expression of BMPER could potentially lead to augmented levels and action of BMPs such as BMP-6, which has been previously shown to inhibit pro-fibrogenic gene expression in activated-HStCs and ameliorate hepatic fibrosis (11). Other BMPs have also been shown to activate demethylation in fibroblasts. Tampe and colleague induced Tet3-mediated hydroxymethylation and demethylation of the RASAL1 promoter by treating activated renal fibroblasts with BMP-7 (321). In a different study by the same research group, demethylation of the RASAL1 promoter by DNMT inhibition with 5aza inhibited EndMT independently of an effect on TET expression (366). The differential demethylation of RASAL1 seen in both studies due to DNMT inhibition or TET-mediated hydroxymethylation could be attributed to differential activation or repression of different molecular pathways or because of the different cell types investigated. Collectively, these studies highlight DNA methylation as an important cellular process in modulating the fibroblast/myofibroblast phenotype and that these established fibrotic

characteristics can be manipulated by pharmacological DNMT inhibition or through activation of intrinsic demethylation processes.

Histone Acetylation in Fibroblasts

Therapeutic intervention or genetic manipulation of the histone acetylation/deacetylation machinery has been shown to prevent the development of fibrosis and contribute to an overall improvement in cardiac function in preclinical models of heart failure (148, 160, 244, 245, 272, 323, 330). These agents are thought to impact the cellular function of fibroblasts due to various reasons including inhibition of proliferation and attenuating their ability to respond to both developmental and injurious cues. The role of HATs and HDACs in fibroblasts is outlined in **Table 10**.

Pan-HDAC inhibitors have demonstrated their anti-fibrotic properties on isolated activated ventricular fibroblasts. Kong and colleagues were able to suppress collagen 1 synthesis and fibrogenesis by *in vitro* treatment with TSA (177). Both HDAC1 and HDAC2 have been previously shown by Nural-Guvener *et al*., to be strongly expressed in fibroblasts in the ischemic myocardium of rats which experienced chronic myocardial infarction for 6 weeks (245). Their work revealed that administration of the selective benzamide class I HDAC inhibitor, Mocetinostat, reduced expression of αSMA and other markers of active myofibroblasts including collagen III and MMP2. Treatment of primary cardiac fibroblasts with Mocetinostat also upregulated p21/p53 and levels of cleaved caspase-3, which are involved in cell cycle arrest and apoptotic responses. Further work carried out by that group has highlighted that the inhibitory action of class I HDACs on activated fibroblasts could be through modulation of the IL-6/STAT3 signalling pathway leading to attenuation of their proliferation, migration and activation (244).Other potent HDAC inhibitors have also been shown to inhibit fibroblast activation. Treatment of primary cardiac fibroblasts with MPT0E014 inhibited cell proliferation, even in the presence of TGFβ and reduced expression of the Angiotensin-II type I receptor, a receptor known to activate a pro-fibrotic and pro-inflammatory fibroblast phenotype upon ligand binding of Angiotensin-II (160).

Similar blunted myofibroblast responses after HDAC inhibition have been indicated from fibroblasts cultured and stimulated from other organ systems including the liver (242, 266), kidney (195), lung (119, 284) and skin (33, 275). To determine the involvement of HDACs in the transdifferentiation of dermal fibroblasts to myofibroblasts with TGFβ, Glenisson and colleagues carried out siRNA knockdown of HDACs followed by activation with TGFβ. They discovered that knockdown of HDAC4, HDAC6, and HDAC8 expression impaired TGFβ-induced αSMA expression, but repression of HDAC4 expression in particular returned levels of αSMA back to control levels (110). This work was supported by Guo *et al*., who showed that HDAC4 knockdown resulted in attenuation of pulmonary myofibroblast activation (119). The mechanism they concluded was that HDAC4 plays a role in myofibroblast activation by regulating TGFβ-induced Akt phosphorylation (119). Other reports have also deemed HDAC4 to be an important regulator of MMP9 and MMP13 expression upon myofibroblast transdifferentiation in hepatic fibrosis (266).

Analysis of these HDAC inhibitors in these fibrotic disease contexts has highlighted putative genes that are abnormally regulated by deacetylation of histone proteins and may be linked to the development of an activated myofibroblast phenotype. Russell *et al*., found that expression of SFRP1, an inhibitor of Wnt signalling, was restored in dermal keloid fibroblasts after treatment with TSA and was associated with a decreased expression of collagen and CTGF/CCN2 (279). Thy-1 expression, as previously described is regulated by DNA methylation in pulmonary fibroblasts and is also modulated by histone deacetylation. Sanders *et al*., found that TSA treatment of fibrotic pulmonary fibroblasts partially restored expression of Thy-1 and aided in demethylation of its gene promoter leading to decreased expression of αSMA (284). This study nicely demonstrates how epigenetic mechanisms interact together to regulate gene expression and function of fibroblasts.

With the majority of work focused on the role of HDACs in driving an abnormal myofibroblast response, evidence on a role of HATs in the promotion of a cardiac myofibroblast phenotype has been limited but is evident in other fibrotic disorders. Fibroblasts derived from the skin of patients with systemic sclerosis express elevated levels of p300 compared to healthy fibroblasts. Furthermore, manipulating p300 levels *in vitro* revealed that Smad-dependent TGFβ activation of fibroblasts is dependent upon p300 (33). Collectively these studies highlight that histone acetylation and deacetylation are clearly important in modulating fibroblast phenotype and function and have the potential to contribute to abnormal cardiac wound healing.

Histone Methylation in Fibroblasts

While it is becoming increasingly evident that changes in histone methylation and demethylation are present in heart failure, the exact contribution of these epigenetic enzymes to modulating the cardiac fibroblast phenotype and promotion of cardiac fibrosis is unknown. Many of the studies investigating the involvement of histone methylation in development of the pro-fibrotic myofibroblast phenotype are centred in other fibrotic disease areas such as hepatic, pulmonary and renal fibrosis, but could shed some light onto the acquisition of the hyperactive myofibroblast phenotype in the injured and failing heart (**Table 11**).

The G9a methyltransferase is evident as being a key player in renal, hepatic and pulmonary fibrosis. TGFβ-induced phosphorylation of Smad3 was found to augment expression of G9a in activated rat interstitial kidney fibroblasts and induce expression of fibrotic markers αSMA and fibronectin (145). These pro-fibrotic changes both *in vitro* and *in vivo* were abrogated by application of the selective G9a-methyltransferase inhibitor BIX01294, with a reduction in repressive H3K9me1 levels (145). Similar therapeutic benefit through H3K9me1 suppression by G9a-methyltransferase inhibition has been also recently described in an *in vitro* model of peritoneal fibrosis (201). Involvement of G9a in gene repression has also been reported in pulmonary fibrosis through H3K9 trimethylation of the anti-fibrogenic cyclooxygenase-2 (COX2) gene promoter (69). Silencing of the COX2 gene in idiopathic pulmonary fibrosis patient-derived fibroblasts was also facilitated by interdependent action of DNA methylation and H3K27 trimethylation by the lysine methyltransferase EZH2 (69). Pharmacological inhibition of G9a-methyltransferase by BIX01294 and EZH2 by 3-Deazaneplanocin A (DZNep) improved the phenotype of pro-fibrotic pulmonary fibroblasts by augmenting the expression of COX2 and its product prostaglandin E2 (69).

Acquisition of the myofibroblasts phenotype by HStCs and development of hepatic fibrosis has been associated with global changes in histone methylation marks, with increased trimethylation of both H3K4 and H3K27 (254). Complementary studies have been carried out to dissect the role of specific histone methyltransferases in creating this fibrotic myofibroblast phenotype. ASH1 methyltransferase has been proven to bind to the promoter regions of pro-fibrotic genes αSMA, TIMP-1, collagen-1 and TGFβ, facilitating active H3K4me3 marks and promoting active gene expression (254). Furthermore, H3K27me3 repressive methylation marks in the 3' exons of PPARy are known to induce transcriptional silencing and are created by the lysine methyltransferase EZH2 in active myofibroblasts (385). Evidently, both genetic knockdown and pharmacological inhibition of EZH2 activity by DZNep reduced fibrotic gene expression, inhibited morphological changes and myofibroblast proliferation (385). It seems that the expression and recruitment of these lysine methyltransferases, and their ability to promote and establish a myofibroblast phenotype and tissue fibrosis, is regulated by MeCP2 (254, 385). Whilst DZNep inhibition of EZH2 shows therapeutic promise, a major problem faced with any epigenetic therapy is to therapeutically target specific cells. Zeybel and colleagues have recently shown promising data in which they have been able to specifically target hepatic myofibroblasts *in vivo* using a C1-3-liposomal vector carrying DZNep (385). Treatment with targeted DZNep displayed improvements in hepatic collagen deposition along with reduced expression of fibrotic markers collagen 1A1, CTGF/CCN2 , and angiopoetin 1 in the hepatotoxic carbon tetrachloride model of hepatic fibrosis (385).

JMJD enzymes facilitate the removal of methyl groups from lysine residues on histone proteins through oxidative demethylation. Expression of several histone demethylating enzymes including KDM2B, JMJD1A, JMJD2B, JMJD2C, JMJD3 and KDM5B has been previously shown to be inducible in hypoxic conditions through HIF-1α (186, 282). This suggests that indeed when the myocardium becomes hypoxic, either due to perfusion defects or increased oxygen consumption by infiltrating inflammatory cells and metabolically active fibroblasts, active lysine demethylation can occur with subsequent regulation of gene expression. Removal of H3K4me3 marks by the histone demethylase KDM2B has been highlighted as an important regulator of fibroblast function. Over expression of KDM2B in murine embryonic fibroblasts was associated with dynamic transcriptomic changes of 264 genes involved in various cellular processes including inflammation (chemokines CCL2, CCL5, CCL7, and CXCL10), metabolic processes (Crabp2 and Ripbk3) and ECM production (Col2A1) (151). Chemokines are signalling molecules that play an important role in the recruitment of inflammatory cells to the site of tissue injury to initiate the wound healing process. Sustained secretion of these signalling proteins by cardiac fibroblasts, due to alterations in histone demethylation, can result in persistent inflammatory cell recruitment to the heart. Consequently, this may exacerbate a vicious cycle of inflammation and hypoxia, and lead to further tissue damage, aberrant wound healing and cardiac fibrosis.

Epigenetic Regulation of Immune Cell Function

Immune cell infiltration into the damaged heart is a major contributor for stimulating cardiac remodeling, due to the release of inflammatory mediators and the phagocytic removal of damaged tissue within the heart. Inflammatory cells rely upon their ability to degrade ECM in order to infiltrate cardiac tissue and also play a major role in driving fibroblast production of new ECM during the wound healing process. Healthy restoration of normal tissue architecture requires a delicate balance between pro-inflammatory, pro-resolution, and pro-wound healing actions of immune cells. The microenvironment of the damaged heart can influence the function of tissue-resident immune cells as well as the recruitment and behaviour of infiltrating immune cells.

Neutrophils are polymorphonuclear granulocytes that play a critical role in innate immunity. They are the first-line immune cells recruited to sites of injury or infection. The role of neutrophils in myocardial infarction is well documented (99). In response to acute cardiac injury, recruited neutrophils become activated by danger-associated molecular patterns (DAMPs) such as liberated intracellular ATP, mitochondrial DNA, heat shock proteins and fibronectin fragments (73, 200). Once activated, neutrophils release their granular contents including ROS, cytokines, chemokines and MMPs (MMP8 and MMP9), which function to drive inflammation and tissue remodeling after injury (200). Whilst it is well established that neutrophils play an important role in tissue responses to acute injury their contribution to aberrant cardiac remodeling in the setting of chronic injury such as that seen in hypertensive heart disease is unclear.

Monocytes are involved in various physiological processes including inflammation and tissue remodeling. They are a major constituent of both the innate and adaptive immune response. Monocytes are derived from bone marrow-derived progenitor cells and are recruited to the site of injury where they differentiate into pro-inflammatory M1 or “classical” macrophages, M2 or “alternative” macrophages or dendritic cells, depending upon specific signalling cues (62, 114). M1 macrophages are involved in pro-inflammatory responses secreting heavy amounts of pro-inflammatory mediators (including TNF-α, IL-1, IL-6, IL-8, nitric oxide and ROS generation), killing intracellular pathogens and driving T-helper (Th)-1 responses. M2 macrophages promote contrasting anti-inflammatory/pro-resolution responses producing cytokines such as TGF-β, IL-10 and vascular endothelial growth factor (VEGF), contain enzymes such as arginase (Arg-1) and are involved in the promotion of Th-2 responses and the killing of extracellular pathogens (111, 114, 208). These two different activated states are extensively distinguished throughout the literature by murine markers such as inducible nitric oxide synthase (iNOS), C-X-C motif chemokine 10 (CXCL10) for M1 macrophages and Ym1, Fizz1, Arg-1, Retnla and mannose receptor (CD206) associated with M2 phenotype (210). It is becoming more appreciated that

there are specific subsets of M2 macrophages such as 2A, 2B and 2C which acquire different functional immunological properties that are microenvironment dependent (207). It is now becoming more apparent that macrophages have a great deal of plasticity regarding their phenotype and have the capacity to switch from one activated state to another (260).

Various subsets of macrophages reside locally within the heart, termed cardiac macrophages. These cells are involved in coordinating cardiac inflammation along with antigen sampling and wound repair (85, 257). Dendritic cells are specialised antigen-presenting cells which link the innate and adaptive immune response. They patrol both the blood and peripheral tissue where they process antigens through phagocytosis and present them to T-cells in lymphoid organs along with supplying cytokines that facilitate T-cell activation and differentiation (20, 21). Monocytes/macrophages and dendritic cells are implicated in many cardiovascular disorders and are well documented as playing a role in atherosclerosis and myocardial infarction (147, 318).

Cells of the adaptive immune system have also been shown to be implicated in chronic inflammatory conditions such as heart failure. T cells are the principal cells involved in adaptive immunity and arise from common lymphoid progenitor cells where they are defined into either CD4+ helper-T cells or CD8+ cytotoxic-T cells on the basis of their co-receptor (CD4 or CD8) which facilitates the binding of T cell antigen receptors to the antigen-bound major histocompatibility complex molecules (218). Naïve CD4+ T-cells undergo proliferation and differentiation into lineage-specific effector cells such as Th-1 cells, Th-2 cells, Th-17 cells, regulatory T (Treg) cells and follicular helper T (Tfh) cells once activated by antigen presenting cells (391, 392). Lineage-specific differentiation of T-cells depends on numerous factors including cytokine milieu, type of antigen presenting cell, concentration of antigen and type of co-stimulatory molecules which activate lineage-defining master regulators that govern T-cell specific cellular phenotypes (135, 391). CD4+ T-cells have previously been shown in animal models to contribute to cardiac remodeling and accelerating disease progression to heart failure (23, 184, 240). In patients, the percentage of CD4+ T cells in blood has been shown to correlate with LV dysfunction (104). Interestingly, specific subsets of CD4+ T-cells can also be altered in chronic heart failure. A study by Tang *et al*., found that patients with chronic heart failure had a significant reduction in the number and functional capacity of circulating Treg cells (which are governed by expression of the master regulator FoxP3) compared to healthy controls, with a correlation between Treg cell dysfunction and disease severity (322).

The ability of immune cells to respond to various injurious and infectious stimuli is critical for homeostasis. While it is well known that immunological memory is a trait of the adaptive immune system, it is becoming increasingly evident that there is the presence of immunological memory within innate immune cells, allowing for these cells to respond appropriately to certain stimuli (239). Two examples of this innate immune memory are trained immunity and immunotolerance (such as endotoxin clearance) (8). Trained immunity refers to when innate immune cells are primed by an initial challenge, allowing for an enhanced immunological response when they experience a secondary challenge. After initial exposure of fungal wall products (such as β-glucan), monocytes can mount an enhanced pro-inflammatory response by augmenting cytokine secretion upon second exposure (267). Immunotolerance involves the suppression of innate immune cells where they enter a refractory functional state and do not respond upon re-stimulation, as is the case with pre-stimulating monocytes with lipopolysaccharide (LPS) (217).

Cellular differentiation into the effector cells of the immune system including both the innate and adaptive systems is accompanied by changes in gene expression. Studies are now highlighting the role of epigenetic modifications as being important mechanisms involved in regulating gene expression for cellular differentiation, governing cellular phenotype and controlling cellular and immunological responses within the tissue microenvironment. Alterations in these cellular processes could result in acquisition of a dysfunctional cellular phenotype which is followed by the promotion of improper cardiac wound healing.

DNA Methylation in Immune Cells

The implication of DNA methylation in dictating immune cell phenotype and cellular function is becoming increasingly apparent (**Table 12**). It is involved throughout the various cellular stages

of haematopoiesis including the transition from haematopoietic stem cells (HSCs) to the various progenitor stages (multipotent progenitors (MPPs), common myeloid (CMP)/lymphoid progenitors (CLP), granulocyte-macrophage progenitors (GMPs)) and finally differentiation into effector cells of the immune system (monocytes, macrophages, granulocytes, T cells, natural killer (NK) cells and B cells) (8, 154). Mice with reduced DNMT1 activity can only produce progenitors for the myeloerythroid lineage and are unable to generate lymphoid progenitor cells (39). The function of *de novo* methylation by DNMT3 enzymes is also important because loss of both DNMT3A and DNMT3B function in HSCs results in impaired cellular differentiation and enhanced capacity for cellular self-renewal (52). TET2 is also a critical regulator of self-renewal and differentiation of hematopoietic stem cells as deficiency of TET2 resulted in delayed HSC differentiation and skewed the development to monocyte/macrophage lineage (172).

DNA methylation is becoming established as an important regulator of neutrophil differentiation, identity and function. Differential methylation analysis of human leukocytes revealed unique methylation signatures between cell subsets derived from either myeloid or lymphoid lineage. Neutrophils were found to have extensive hypomethylation compared to CD8+ T cells (153, 396). Furthermore, dynamic changes in DNA methylation are essential during stages of neutrophil differentiation and maturation. Ronnerblad and colleagues found reduced CpG methylation mirrored increased gene expression of both transcription factors (PU.1 and GFI1) and granule proteins (myeloperoxidase, elastase, and proteinase 3) in promyelocyte/myelocytes (granulocyte precursor cell) (276). Paradoxically, at the gene loci, mature neutrophils display a similar degree of hypomethylation at the same gene loci but their expression is markedly reduced (356). One proposed mechanism for this reduced gene expression in healthy mature neutrophils is by Runt-related transcription factor 3 (RUNX3) (65). Aberrant neutrophil expression of myeloperoxidase and proteinase 3 has been demonstrated in neutrophils of patients with anti-neutrophil cytoplasmic autoantibody (ANCA) vasculitis (65). Interestingly, neutrophils from patients with ANCA vasculitis have a reduced expression of RUNX3 associated with increased DNA methylation at the RUNX3 promoter (65). This data highlights the role of DNA methylation in regulating inflammatory neutrophil function and is potentially relevant in the setting of cardiac injury. Regulation of CD4 and CD8 expression is critical for T cell identity. T cells undergo multiple rounds of cell division after activation and therefore it is crucial that expression is finely tuned through heritable cellular processes to dictate the correct phenotype. Sellars *et al*., reported that methylation of the CD4 locus by DNMTs is required to maintain CD4 silencing in cytotoxic CD8+ T cells and that stable expression of CD4 in CD4+ T cells is dependent on the cis-acting enhancer E4P-directed active DNA-demethylation (293). Differentiation of CD4+ into subset-specific T-cells is also linked to DNA methylation including Th1 (289, 359), Th2 (155), Th17 (233, 375) and Treg cells (288, 399) as methylation is an underlying mechanism that facilitates the control of subset-specific T-cell gene expression. Treg cells have essential immunosuppressive functions and their absence due to mutations of the FoxP3 gene is known to result in the development of systemic autoimmunity (395). Thymically-derived Tregs are known as natural Tregs (nTreg) whereas Treg cells that arise from naïve CD4+ cells in the periphery in response to self or non-self-antigens and are dependent on TGFβ are known as induced-Treg cells (iTreg). DNA methylation has been demonstrated as a potential factor for distinguishing these two subsets of Treg cells through modulation of FoxP3 (94). Upstream of the FoxP3 promoter lies a conserved CpG-rich region denoted as a Treg-specific demethylated region (167). This region is demethylated in nTreg cells with stable FoxP3 expression whereas in iTreg cells there is residual methylation in this region, resulting in less stability in its expression (135, 143). Furthermore, it is becoming clearer that DNA methylation may also play a role in the memory of T cells with differential gene methylation apparent between naïve and memory CD4+ T cells, enabling the memory cells to be primed and rapidly respond upon activation compared to naïve cells (175).DNA demethylation seems to be a major modification involved in the transition of monocytes into either macrophages or dendritic cells (170, 389). Demethylation occurring in the differentiation of monocytes to macrophages has been shown to occur within the first twelve hours of stimulation with recombinant human monocyte colony-stimulating factor (M-CSF) (339). The involvement of TET2 in demethylation was demonstrated by use of the competitive dioxygenase inhibitor 2α-hydroxyglutarate (2-HG), which was associated with increased

methylation levels, preventing *in vitro* morphological changes (cellular protrusions) and adherence (339). The authors demonstrated, using high throughput sequencing, that DNA demethylation occurs in distant enhancer regions in genes involved in signalling pathways which regulate the actin cytoskeleton and phagocytosis upon differentiation of monocytes to macrophages (339). On the other hand, *ex vivo* differentiation of dendritic cells from monocytes was associated with 1,608 different methylation points (1367 of these demethylated), with only 6% of these points located within CpG islands and the rest occurring at non-CpGI and transcription factor binding sites. These changes in methylation throughout the genome were associated with alterations in expression of DNMT enzymes (DNMT1 and DNMT3A) and TET2 (389). Demethylation of CpGs in the promoter region of CD209, a dendritic cell specific marker, was shown to occur during the transition from monocyte to dendritic cells (41). Vento-Tormo and colleagues have shown that differentiation of monocytes to dendritic cells through IL-4 stimulation results in activation of JAK3-STAT6 pathway, and promotes specific demethylation and subsequent activation of a subset of dendritic cell genes along with repression of macrophage specific genes (334). Interestingly, a recent study by Pacis *et al*, has demonstrated that monocyte-derived dendritic cells undergo dynamic changes in DNA methylation at distal enhancer elements when infected with mycobacterium tuberculosis (251). Regions that are hypomethylated were enriched near genes known to play a key role in the regulation of immune processes and were associated with genes differentially expressed in response to infection, implicating the role of DNA methylation in dendritic cell function (251).

As DNA methylation plays an important role in regulating macrophage phenotype and function, alterations in the methylating machinery as a result of pathological conditions that contribute to heart failure development may result in an abnormal cellular behaviour. Indeed, DNMT3B expression has previously been shown to be significantly elevated in macrophages isolated from adipose tissue of *ob/ob* obese mice compared with those isolated from lean controls (379). *In vitro* knockdown of DNMT3B in Raw264.7 cells resulted in polarisation towards the M2 phenotype with expression of M2 markers (Arg1, mannose receptor C type 1, and macrophage galactose-type c-type lectin) and profoundly reduced LPS- and stearate-induced inflammatory gene expression (TNF-α, IL-1β and iNOS) (379). DNMT3B repression-induced M2 polarisation seems to be related to the methylation status of the CpG rich 5' untranslated region of the PPARγ1 gene. Pyrosequencing analysis carried out by Yang *et al*, revealed that stimulation of macrophages with stearate increased DNA methylation at PPARγ1, which was significantly decreased by DNMT3b knockdown (379).

Recent studies have also demonstrated the importance of the maintenance methylating enzyme DNMT1 in regulating macrophage phenotype and inflammation in various disease settings including obesity and atherosclerosis (345, 381). Wang *et al*., demonstrated that macrophages isolated from the adipose tissue of *ob/ob* mice had a higher expression of DNMT1 and M1 proinflammatory markers compared to lean controls (345). They also found that mice with a myeloid-specific deletion of DNMT1 fed a high fat diet exhibited an increased presence of M2 macrophages in the adipose tissue along with a reduction in basal and LPS-stimulated IL-6 and TNF-α expression from macrophages isolated from both adipose tissue and bone marrow (345). DNMT1 involvement in inflammatory cytokine release from macrophages has also been demonstrated in atherosclerosis (381). Macrophages from transgenic mice with macrophage-specific over-expression of DNMT1 were found to have increased circulating plasma levels of TNF-α, IL-6 and IL-1β which promoted atherosclerosis development (381). For both these studies, the role of DNMT1-mediated macrophage phenotype and inflammation seems to be driven by increased methylation of the PPARγ proximal promoter, especially in response to pro-inflammatory cytokines and saturated fatty acids (345, 381). Decreased PPARγ gene expression was found to be mirrored with an increased expression of DNMT1 and pro-inflammatory cytokines in monocytes isolated from patients with atherosclerosis (381). Other potential targets for DNMT1 enabling the promotion of macrophage inflammation include the SOCS1 gene promoter. Hypermethylation of SOCS1 promoter during LPS activation of macrophages resulted in the prolonged secretion of TNF-α and IL-6 (60), two major cytokines which correlate with disease severity in heart failure.

Two common comorbidities associated with the development of heart failure are hyperglycaemia in diabetes and hyperlipidemia in obesity. Babu *et al*., carried out genome-wide DNA methylation sequencing to investigate alterations in DNA methylation in the proximal promoter regions of tissue macrophages isolated from hyperglycaemic and hyperlipidemic mouse models of hindlimb ischemia (15). They identified 198 genes in the hyperlipidemia model and 272 genes in the hyperglycaemic model which showed proximal promoter hypomethylation. Hypermethylation of proximal promoters was found in 102 genes in the hyperlipidemia model and in 136 genes in the model of hyperglycaemia (15). Collectively this shows altered proximal promoter methylation in macrophages found in models of metabolic dysfunction. Promoter methylation was shown to influence expression of macrophage subset-specific genes with hypermethylation and repression of M2 genes (Plxnd1, Nrp1, Cdk18 and Fes) and hypomethylation of M1 genes (Cfb, Serping1 and Tnfsf15) in macrophages isolated form ischemic muscle. Both methylation and expression analysis seemed to confirm the presence of a dominant pro-inflammatory macrophage phenotype within ischemic muscle with repression of the M2 phenotype in these models, confirmed by immunohistological assessment for TNF-α. This persistent M1 phenotype was not observed within the control ischemic muscle (15). Dysregulation of DNA methylation in the context of metabolic dysfunction could result in the abnormal persistence of the pro-inflammatory macrophage phenotype. Interestingly, a long term follow up study of type 1 diabetic patients has recently demonstrated that persistent differential DNA methylation at key genomic loci was detected in both whole blood and monocytes is associated with diabetic complications (59). Considering that monocyte populations are altered in different types of heart failure (24, 112) and have the potential to differentiate into macrophages or dendritic cells in response to injury, these aberrant stable changes in DNA methylation in the setting of metabolic dysfunction may explain why comorbidities such as obesity and diabetes contribute to aberrant inflammatory responses and the development of cardiomyopathy.

Pharmacological intervention of DNMT activity has been shown to modify both cellular phenotype and function. Acquisition of FoxP3 expression, a marker of Treg cells, can be achieved in NK cells when stimulated by IL-2-induced STAT signalling but only in the presence of 5azadC, suggesting the reprogramming of cellular identity through DNMT inhibition (399). 5azadC can also induce transition of CD4+ Tcells into Treg cells *ex vivo* without the presence of TGFβ and has demonstrated beneficial effects in cardiac allograft survival upon adoptive transfer of these transformed cells in combination with continuous 5azadC treatment (61). Dendritic cells derived from monocytes and activated *in vitro* in the presence of 5aza were found to secrete lower levels of IL-10 and IL-27 cytokines (102). With these changes seen *in vitro*, Frikeche and colleagues followed up to investigate if these changes in cytokine secretion by activated DCs could influence T cell responses *in vivo* in a small cohort of MDS patients who received 5aza treatment. Patients who were treated with 5aza showed promotion of Th-17 response showing a significant reduction in IL-4−secreting CD4+ T cells but an increase in both IL-17A−secreting CD4+ T cells and IL-21−producing CD4+ T cells (102), suggesting that inhibition of DNMT can influence expression of genes that are normally silenced, modulating cellular phenotype and identity.

Interestingly, the precise role of Th17 cells in heart failure is unclear. Certain studies have demonstrated augmented levels of Th17 cells and their relevant cytokines to be associated with increased disease severity (191, 235), while others have failed to show any significant difference in heart failure patients (394). Disease association could be due to alterations in the balance between anti-inflammatory Treg cells, known to be reduced in HF, and Th17 cells. As a result, modulating T cell phenotype and function may have the potential to restore balance and prevent inappropriate inflammatory responses.

DNMT inhibitors seem to demonstrate beneficial effects in animal models of cardiovascular disease. In atherosclerosis, $Ldlr^{-/-}$ mice which were treated with 5azadC showed reduced atherosclerotic development independent of atherogenic factors including circulating plasma lipid and cholesterol levels and lipid content of the plaques (46). Macrophage content was found to be decreased in plaques of 5azadC treated mice along with decreased inflammatory gene expression (TNF-α, IL-1β, IL-6 and iNOS) in macrophages isolated from $Ldlr^{-/-}$ treated mice. The atheroprotective effect seen with 5azadC on macrophages was mirrored by reduced methylation

at the promoter region of LXRα and PPARγ with increased gene expression (46). Similarly, 5azadC was also found to have a beneficial effect in obesity through effects on PPARy expression. *Ob/ob* mice treated with 5azadC were found to have an improvement in glucose levels and insulin sensitivity, associated with an increased presence of M2 macrophages in adipose tissue (345). Macrophages isolated from the adipose tissue of *ob/ob* mice treated with 5azadC were found to have an increased expression of PPARy compared to their saline treated littermates. Reducing the inflammatory potential of macrophages with 5azadC has also been demonstrated by preventing SOCS1 hypermethylation (60). Modulation of macrophage phenotype with 5aza in the context of myocardial infarction is associated with therapeutic benefit, reduced cardiac fibrosis and improvement in cardiac function (152, 168). Treatment of rats with 5aza was shown to reduced infiltration of macrophages into the infarcted area, with a more prominent M2 phenotype being present in the 5aza treated hearts compared to an M1 phenotype seen in control animals (168). Modulation of macrophage phenotype in myocardial infarction by 5aza has been recently associated with transcriptional activity of interferon regulatory factor-1 (IRF1) (152). 5aza was shown to maintain levels of IRF1, a transcription regulator of iNOS, in macrophages by preventing proteasomal degradation through sumoylation and therefore repressing the pro-inflammatory M1 phenotype (152).

It is critical that we must also acknowledge the limitations of *in vitro* findings that are based on commercial versus primary cell lines with regard to results from pharmacological DNMT inhibition. It was recently highlighted by Wang and colleagues using bisulfite conversion followed by pyrosequencing that differential basal methylation exists at the PPARy promoter between the commercial Raw 264.7 cell line (33-36% methylation rate) and primary isolated BMDMs (3% methylation rate) (345). While methylation of the PPARy gene was found to increase dramatically in BMDMs in response to inflammatory stimuli (345), the high basal methylation rate found in commercial cell lines such as the Raw264.7 may impact the *in vitro* effects seen with DNMT inhibition. As a result, this difference in methylation status between commercial versus primary cells should definitely be considered for the selection and design of future *in vitro* inflammatory cell experimentation looking at the effect of DNA methylation.

Histone Acetylation in Immune Cells

Understanding the molecular mechanisms that modulate immune cell phenotype and inflammatory gene expression is hoped to provide novel therapeutic avenues for inflammatory conditions. The contribution of acetylation and deacetylation of histone proteins to inflammation is starting to become apparent in various inflammatory conditions including rheumatoid arthritis (64), inflammatory lung diseases (25) and multiple sclerosis (91). Therefore, it is no wonder that acetylation and deacetylation contribute to inflammation in cardiovascular diseases (347). Numerous preclinical models of heart failure have demonstrated improvements in cardiac function after therapeutic manipulation of histone acetylation and deacetylation while also displaying beneficial effects on reducing inflammation (47, 148, 248, 361). While the exact mechanism behind the therapeutic reduction of inflammation in these models remains unknown, one possible mechanism could be through regulation of immune cell phenotype and cellular function.

Polarisation into specific macrophage phenotypes has been demonstrated to be influenced by acetylation and deacetylation (**Table 13**). It has been shown that histone modifications can participate in controlling macrophage function at both promoter and enhancer regions so that they are primed and ready to respond to various stimulating factors in their microenvironment (108). For example, IFN-γ has been shown to prime macrophages that were purified from PBMCs by recruiting transcription factors (STAT1 and IRF-1) and HATS (p300 and CBP), increasing acetylation at promoter and enhancer regions of inflammatory cytokines TNF, IL6 and IL12B to augment their expression when exposed to LPS (265).

As previously mentioned by Kapellos and Iqbal, only a limited number of studies have demonstrated the involvement of HATs in regulating M1 or M2 associated genes (161). A study by Feng *et al*., highlighted the role of p300 in binding to the promoter regions of IFNA, TNF-α and

IL-6 with IRF5 in response to viral infection (92). The majority of the studies have looked at the role of HDACs on regulating macrophage polarisation. HDAC3 has been previously portrayed in the literature as a key regulator involved in macrophage polarisation and inflammatory cell function (58, 234). Deletion of HDAC3 in macrophages, under the control of the myeloid-specific Lysozyme M promoter, facilitated a gene expression profile similar to an M2 phenotype (234). Treatment of HDAC3-deficient macrophages with IL-4 or IL-13 markedly potentiated the induction of genes associated with M2 activation (including Arg1, Clec7a, Ym1, and Retnla) (234). It is now appreciated that the ETS family of transcription factors (including PU.1), which have long been known to participate in macrophage development (292), have the ability to interact with and recruit chromatin remodeling enzymes and other transcription factors in response to stimuli in order to fine tune macrophage gene expression (108, 130). HDAC3 was found to bind to PU.1-guided enhancer regions and through deacetylation, functions to suppress expression of IL-4 target genes and therefore sequestering M2 cellular phenotype (234). The influence of HDAC3 in promoting pro-inflammatory macrophage phenotype was confirmed by Chen and colleagues, where they found that HDAC3 is required for inflammatory gene expression in response to LPS through IFN-β production as this allows for the maintenance of STAT1 expression (58). In the context of atherosclerosis and inflammation, HDAC3 was previously found to be overexpressed in human atherosclerotic lesions whereby immunohistochemical analysis revealed co-localization of HDAC3 to CD68+ cells in atherosclerotic plaques (136). Interestingly, macrophage-specific deletion of HDAC3 was found to induce an athero-protective macrophage phenotype improving plaque stability (136). Other studies have also demonstrated modulation of macrophages through pharmacological inhibition of HDAC3, where HDACi reduced macrophage apoptosis and expression of IL-6 and NO in response to pro-atherogenic molecules (oxidized Low-density lipoprotein (LDL) and 7-ketocholesterol) (136).

Other HDACs have also been involved in regulating macrophage inflammatory response including HDAC5, 7 and 9. Previous *in vitro* analysis has demonstrated the regulatory role of HDAC5 with regard to cytokine production in both human and murine monocyte/macrophage cell lines (259). Over expression of HDAC5 resulted in increased NF-κB activation along with augmented levels of both pro-inflammatory cytokines (TNF-α and MCP-1) and anti-inflammatory cytokines (IL-10) (259). Significant reduction of TNF-α and MCP-1 expression was seen after knockdown of HDAC5, which was found to be independent of NF-κB activation (259). HDAC6 is also implicated in driving a pro-inflammatory macrophage phenotype as suppression of HDAC6 levels and its activity significantly impaired LPS-induced macrophage activation (371). HDAC7 levels have previously been shown to be elevated in thioglycolate-elicited peritoneal macrophages where an alternatively-spliced isoform of HDAC7 (lacking the first 22 amino acids at the N-terminus) is involved in promoting the expression of a subset of proinflammatory genes in macrophages through interaction with HIF-1α (296). HDAC9 expression was found to be increased in macrophages differentiated from bone marrow-derived cells and THP-1 monocytes (45). HDAC9 deficiency in macrophages resulted in decreased LPS-induced inflammatory gene expression, improved cholesterol efflux and polarisation toward the M2 phenotype associated with increased histone acetylation at the promoters of ABCA1, ABCG1, and PPAR-γ (45). The role of HDACs, including class III/sirtuins, in promoting macrophage phenotype and function has been reviewed previously (71, 161).

Dynamic changes in histone acetylation can contribute to the immunological response of innate immune cells. A study by Saeed *et al*., has demonstrated dynamic changes in histone acetylation upon differentiation of monocytes to macrophages and how these changes can participate in specific innate immune responses i.e. trained immunity (with β-glucan stimulation) versus endotoxin tolerance (with LPS stimulation) (281). The authors identified four epigenetically marked clusters that reflect general transcriptional regulatory regions and are differentially modulated in trained and tolerant immune responses. They found that H3K27 acetylation levels changed at thousands of promoters and distal regions, with acetylation levels correlating well with changes in gene expression (281).

HDACs are crucial for normal immune cell development and function. HSCs that are deficient in HDAC3 have impaired capacity for self-renewal due to defects in DNA replication (311).

Differentiation of HSCs to lymphoid progenitor cells is also impaired in HDAC3-deficient HSCs resulting in a dramatic loss of B and T cells (311). HDAC3 is also essential for the correct and efficient maturation and positive selection of CD4+ and CD8+T cells (310). Now while deacetylation is essential for normal cell development during the progenitor stage, its role in cells which have undergone subsequent differentiation and have less plasticity could be relevant in promoting aberrant phenotypes in cells of the adaptive immune system. Pharmacological inhibition of HDAC activity has been highlighted to have numerous effects on dendritic cell differentiation (56, 238, 312) and function (103) including attenuated expression of co-stimulatory molecules (38, 193, 271), cytokine secretion (188, 238, 305) and modulating antigen uptake and antigen-specific immune responses (38, 193, 271, 305). One of the proposed mechanisms by which HDACi demonstrate their anti-inflammatory potential is through generation of Treg cells (326). Indeed, in the context of pathological cardiac remodeling, adoptive transfer of Treg cells was shown to blunt Angiotensin-II–induced cardiac hypertrophy and fibrosis, independent of any effect on blood pressure (181). It is well understood that both HATs and HDAC are involved in promoting and maintaining the functional Treg phenotype (122). Acetylation by HATs including p300 and TIP60 has been shown to maintain Treg viability and cellular phenotype through modulation of FoxP3 by preventing its degradation and increasing its ability to bind DNA, promoting transcriptional activity (197, 333). Deacetylation by HDAC enzymes has also been implicated in negatively regulating Treg identity and function (6), and has been demonstrated by genetic and pharmacological targeting of HDAC3 (342), HDAC6 (28, 76, 197), HDAC9 (28, 77, 326), and Sirt1 (28) (**Table 14**).

New efforts are underway to develop novel HDAC inhibitors that are specifically designed to target cells of monocytic lineage in order to reduce systemic exposure. Tefinostat (CHR-2845) is a cell-permeant ester that gets cleaved by the human carboxylesterase-1, hCE1 (which is only present in hepatocytes and cells of monocytic lineage) to an acid which accumulates within these cells as it is impermeable due to its charge (383). Tefinostat has demonstrated early signs of efficacy and was found to be well tolerated with an absence of significant toxicity in a phase I clinical trial in patients with advanced haematological malignancies (NCT00820508) (249). This drug is currently in a single-arm Phase 2 study of monotherapy in chronic myelomonocytic leukaemia (MONOCLE) and in a Phase I/II dose escalation trial for cancer associated inflammation in hepatocellular carcinoma (NCT02759601). While more information will be needed to investigate how specific and effective these new class of myeloid-specific HDAC inhibitors are, the potential to selectively target monocytic cells could have translational potential in inflammatory conditions such as heart failure.

Histone Methylation in Immune Cells

Evidence suggests that modulation of immune cell phenotype and function is attributed to both methylation and demethylation of histone proteins. Like other epigenetic processes mentioned, addition and removal of methylated histone marks through transcription factor recruitment is essential for regulating cellular differentiation of immune cells including HSCs (70), progenitor cells (70), dendritic cells (355) and CD4+ T-cells (127, 358). Further highlighting the important role of histone methylation machinery in maintaining normal leukocyte biology is that alterations in these enzymes are associated with human haematological malignancies (2, 58, 150).

Polarisation of macrophages into a particular activated state can be driven by modulation of histone methylation. Studies have demonstrated the importance of histone demethylase enzymes in characterizing the M1 and M2 macrophage phenotype. One histone demethylase that has been identified as a prominent regulator of the M2 phenotype is the JMJD3 H3K27 demethylase, which catalyzes demethylation of H3K27me3 to H3K27me1. M2 marker genes (Arg1, Ym1 and Rentla) were shown to be increased through IL-4/STAT6-upregulation of JMJD3 and subsequent reduction of H3K27me3 methylation at their gene promoters (146). Involvement of JMJD3 demethylase was further supported using the *Schistosoma mansoni* egg challenged *in vivo* model of M2 activation (146). Other studies have also demonstrated the regulation of the M2 phenotype by JMJD3 in response to stimuli such as M-CSF and chitin (286). While Satoh *et al*.,

did not find differences in H3K27 methylation at the M2 gene promoters (Arg1, Ym1 and Rentla) in JMJD3 deficient M-CSF stimulated bone marrow-derived macrophages (BMDMs), they did demonstrate that IL-4 could still up-regulate M2 associated genes in these cells independently of JMJD3 (286). Up-regulation of M2 markers (Arg1, Ym1, Fizz1 and CD206) in response to chitin and M-CSF was found to be dependent on JMJD3 demethylation of H3K27 at the Irf4 promoter, enhancing its transcription and M2 marker gene expression (286). Alternatively, JMJD3 has been implicated in promoting the expression of pro-inflammatory mediators (IL-23p19, TREM-1 and G-CSF) in BMDMs through increasing H3K27 promoter demethylation in response to serum amyloid A (373). Demonstrating that regulation of macrophage phenotype by histone demethylase enzymes is ultimately dependent on the context of the microenvironment, as is the case for certain histone demethylases that have been shown to be induced by HIF-1α in hypoxia (376). More recently it has been highlighted that alterations in histone methylation in bone marrow progenitor cells may influence the inflammatory macrophage phenotype and impair wound healing in T2DM (105). Gallagher and colleagues have demonstrated increased levels of JMJD3 and expression of proinflammatory cytokines such as IL-12 in bone marrow-derived progenitor cells and macrophages in both human and preclinical models of T2DM (105). Increased expression of IL-12 was mirrored by decreased H3K27me3 at the IL-12 gene promoter (105). Consequently, inhibition of JMJD3 activity using the selective H3K27 demethylase inhibitor, GSK-J4, resulted in a dose dependent decrease in proinflammatory gene expression in response to LPS (IL-12, IL-1B and IL-6) (105). Elevated levels of JMJD3 have also been linked to reduced H3K27me3 methylation and increased expression of myeloperoxidase and proteinase 3 in patients with ANCA vasculitis (65).

Methyltransferases have also been implemented in the regulation of macrophage polarisation and function. In response to acquiring the M1 phenotype, there is upregulation in the levels and activity of H3K4-specific methyltransferase KMT2A, which was found to increase H3K4me3 in the promoter region of CXCL10 (169). Pre-treatment of macrophages with MI-2-2, a small molecule inhibitor preventing the interaction between KMT2A and its cofactor menin, resulted in a reduction in the number of CXCL10 positive cells (169). Other lysine methyltransferases such as KMT2B have also been previously linked to governing macrophage responses to microbial stimuli. Deficiency in this methyltransferase prevents H3K4me3 methylation at the transcription start site of the Pigp gene and results in an impaired macrophage response to LPS (12). The role of different histone methylating and demethylating enzymes in contributing to macrophage phenotype and function are reviewed further elsewhere (161).

As highlighted previously, innate immunological memory is achieved by cellular processes that modulate gene expression, with methylation of histone proteins being a key regulator. Demonstration of histone methylation in trained innate immunity was described by Quinten and colleagues whereby they primed monocytes with a low dose of β-glucans from *C. albicans* and found an enhanced production of inflammatory cytokines (TNF-α and IL-6) upon second stimulation with various infections stimuli (267). They found that innate immunological training after β-glucan treatment was attributed to global increases in H3K4me3 marks, with an increased presence at the promoter regions of proinflammatory genes such as TNF-α, IL-6, and IL-18 that strongly correlated with their increased expression (267). Interestingly the authors found increased gene expression of other histone methyltransferases after innate immune training such as SETD7, which has been previously implicated in promoting pro-inflammatory gene expression in monocytes (192). The work carried out by Foster and colleagues demonstrated the role of histone methylation in modulating different classes of TLR-induced genes to achieve immunotolerance in macrophages in response to LPS (96). These two classes of genes include tolerant (such as pro-inflammatory cytokines e.g. IL-6) and non-tolerant genes (such as formyl peptide receptor 1) with their regulation being attributed to the transcriptionally active H3K4me3 mark at their promoters (96). When tolerant macrophages were stimulated with LPS, H3K4me3 would be selectively lost in tolerant genes but maintained on non-tolerant genes. This was confirmed using an inhibitor of H3K4 demethylase LSD1, which prevented IL-6 silencing in tolerant macrophages (96). The maintenance of H3K4me1 marks at latent enhancer regions of the genome, deposited in response to stimuli such as LPS, have also been proposed to

contribute to innate immunological memory and macrophage function, even after the stimulus has been removed (250).

Histone H3K9 methylation has been shown to play a role in the regulation of dendritic cell inflammatory responses. Upon LPS treatment, demethylation of H3K9 and recruitment of RNA polymerase II was associated with activation of pro-inflammatory gene expression in dendritic cells (280). Recruitment of transcriptional machinery was shown to correlate with H3K9 demethylation rather than acetylation of H3 and H4 proteins (280). Furthermore, dendritic cells show significantly lower levels of H3K9me2 and H3K4me3 at both IFN and IFN-inducible genes suggesting that these cells are epigenetically programmed to respond to inflammation (89). Of note, Prakash and colleagues have demonstrated impairment of IFN secretion by monocyte-derived dendritic cells from aged individuals in response to the influenza virus. This age-associated decline in dendritic cell function was associated with increased H3K9me3 in non-stimulated aged-DCs and decreased H3K4me3 marks upon viral stimulation in the promoter regions of IFN and IL-29 genes (263).

The microenvironment can influence naïve CD4+T-helper cell differentiation into various T-helper cell responses including Th1 and Th2. Cytokines can activate various signalling pathways including STAT proteins, allowing for emergence of specific T-helper cell subsets (189). Regarding IL18rap gene expression in T-helper cells, STAT4 (activated by IL-12) promotes permissive marks such as H3K4me3 in Th1 cells, promoting IL18rap gene transcription, whereas STAT6 (activated by IL-4) is critical at establishing repressive H3K27me3 marks at the same locus of the IL18rap gene in Th2 cells (354). This demonstrates the ability of STAT proteins to modulate histone methylation at the same genomic loci creating opposing function allowing gene regulation for T-helper cell differentiation (354). Regulation of FoxP3 expression in Treg cells has also been attributed to histone methylation. It was found that Treg cells lose their FoxP3 expression (FoxP3-losing cells) during *in vitro* expansion (noticeable at 2 weeks) with down-regulation of SOCS2, a protein involved in maintaining FoxP3 stability (171), mirrored with increased expression of Th2-lineage genes such as GATA3, GFI1, IL-13 (126). In FoxP3-losing cells, H3K4me3 marks (associated with active transcription) were absent at the FoxP3 promoter but were found at considerably higher levels in IL-4 and IL-5 genes, correlating with their increased expression. Interestingly there was no difference in H3K4me3 levels in the GATA3 gene between FoxP3+ cells and FoxP3-losing cells and the authors speculate that it could be due to a complex relationship between FOXP3 and GATA3 (278) and these permissive H3K4me3 marks provide a prerequisite for GATA3 up-regulation in these cells for T-cell plasticity (126).

Th17 differentiation has also been shown to be highly regulated by histone methylation. Jumonji- and AT-rich interaction domain (ARID)-domain-containing protein 2 (JARID2) is a DNA-binding protein that interacts with PcG2 to negatively regulate Th17 gene expression through H3K27 methylation (87). miR-155 is highly expressed in Th17 cells and has been shown to negatively regulate expression of JARID2 (87), highlighting the complex nature of non-coding RNA influencing epigenetic regulation of histone methylation to facilitate Th17 differentiation.

Problems Faced With Targeting Inflammation in Heart Failure

With inflammation being a hallmark of cardiac injury, it was presumed that agents that target inflammatory mediators released by cells of the immune system may yield a beneficial form of therapy for the treatment of heart failure. Previous approaches investigated include antagonizing the effects of inflammatory cytokines such as TNFα. Two strategies were employed for antagonizing the actions of TNFα such as using soluble decoy receptors (Entanercept) and using chimeric monoclonal antibodies (Infliximab) to neutralize the effects of TNFα. These were investigated in large scale multi-centre clinical trials; The Randomized Etanercept North American Strategy to Study Antagonism of Cytokines (RENAISSANCE, n=900 patients) trial, Research into Etanercept Cytokine Antagonism in Ventricular Dysfunction (RECOVER, n=900 patients) trial and Randomized Etanercept Worldwide Evaluation (RENEWAL, n=1500 patients) investigated the therapeutic potential of Entanercept and the phase II trial Anti-TNFα Therapy Against CHF (ATTACH, n=150 patients) examined the use of infliximab in moderate to advanced heart failure

(202, 203) While these agents are used clinically for the treatment of inflammatory conditions such as rheumatoid arthritis, these trials failed to demonstrate any therapeutic benefit in heart failure patients. The trials also revealed that anti-TNFα therapy adversely affected the course of the disease with all three trials demonstrating increased all-cause mortality and hospitalizations with the anti-TNFα treatment groups (18, 67, 204). The exact reasons for the adverse outcome of anti-TNFα therapy seen in heart failure remain unclear; however one plausible mechanism for the detrimental outcome that has been alluded to is that anti-TNFα therapy (e.g. Infliximab) binds to TNFα expressed on the sarcolemma of failing cardiac myocytes causing cellular apoptosis through complement fixation, promoting disease progression and cardiac failure (139, 203). Perhaps the focus should be on targeting the inflammatory cells themselves rather than targeting the molecules they produce, either by inhibiting their recruitment to the heart or by modulating their inflammatory potential in the heart directly. While targeting local inflammation directly in the injured heart is an extremely challenging obstacle, targeting the activation, mobilization and recruitment of pro-inflammatory cells to the heart might enable a more promising therapeutic window. Recently, in ischemic cardiomyopathy, the spleen has been implicated as a source of inflammatory cells including monocytes, macrophages, dendritic cells (pDCs and cDCs) and CD4+ T cells, which possess an activated pro-inflammatory state that is maintained upon adoptive transfer and can induce pathological cardiac changes in naïve animals (23, 147). While the reticuloendothelial role and microanatomy of the spleen has been a major challenge for effective delivery and pharmacokinetics of micro and nanoparticle based compounds (50), this could be utilized as an advantage to deliver targeted therapy specifically to specific pro-inflammatory cell populations before they mobilize to the heart. Indeed compounds already in clinical use have been shown to readily accumulate in cells of the spleen such as Clofazimine, an antibiotic and anti-inflammatory drug used for the treatment of leprosy and skin inflammation that has been shown to be readily taken up and stored by macrophages in the spleen (17). Delivering anti-retroviral treatment specifically to splenic macrophages has also become an attractive target for the treatment of HIV (270). While the specific antigenic activation that promotes the pathological change in splenocytes in ischemic heart failure is unknown, epigenetic mechanisms could be involved in retaining their pathological cellular memory. This could therefore act as a novel therapeutic window to directly modulate inflammatory cell phenotype and function with epigenetic modifying therapies.

Conclusion

The cardiac response to injury involves a complex interplay of tissue resident cells such as endothelial cells, fibroblasts, cardiomyocytes, and immune cells, and infiltrating inflammatory and mesenchymal precursor cells. Physiological adaptation to injury or stress requires the maintenance of tissue integrity, via appropriate ECM deposition, cardiomyocyte hypertrophy in order to maintain cardiac output, and adaptation of the vasculature in order to support myocyte function. Physiological adaptation is followed by resolution of inflammation and myofibroblast function, remodeling of ECM to match the degree of strain in the myocardium, and stabilisation of new blood vessels. In pathological settings either persistent injury, exaggerated inflammatory or fibrotic responses, inadequate angiogenic responses, or uncontrolled myocyte hypertrophy, or quite possibly a combination of a number of these events, leads to aberrant wound healing and cardiac dysfunction. Physiological responses to injury are complex and clearly require carefully controlled changes in cellular phenotypes, in both resident and infiltrating cells, which are dictated by altered transcriptional profiles. The persistence of activated inflammatory cells, activated myofibroblasts and hypertrophic cardiomyocytes during aberrant wound healing situations suggests the involvement of epigenetic mechanisms that lead to the survival of cells with stable phenotypic changes.

The role of epigenetic changes in the pathogenesis of heart failure is increasingly apparent; however the majority of our knowledge has come from studies utilizing cardiac tissue from patients and animal models, which contains a mix of different cell types, or *in vitro* cell culture systems. It is clear that epigenetic changes can occur in different cell types, at different rates, at

different sites and can have differential effects depending on the cellular context. *Ex vivo* isolation of individual cell types from injured myocardial tissue, and subsequent analysis of epigenetic modifications, is greatly improving our understanding of disease-relevant alterations in chromatin structure and transcriptional activity. The goal of this overview was to highlight the specific changes in DNA methylation, histone acetylation and methylation which independently contribute to an aberrant cellular phenotype, focusing specifically on cells that are involved in cardiac dysfunction such as cardiomyocytes, fibroblasts and immune cells. It is becoming increasingly apparent that endothelial cells play an important role in the pathophysiology of heart failure. Both DNA methylation and histone modifications have been previously shown to modulate endothelial cell-specific genes (such as eNOS, Von Willebrand factor and CD31) (97, 302) and govern essential biological processes central to the wound healing response such as angiogenesis, inflammation and response to blood flow (212). While not covered as part of this review, the regulation of endothelial cell gene expression by DNA methylation and histone modifications has been reviewed elsewhere (97, 212, 372).

Remarkable progress has been made in our understanding of the ability of DNA methylation and histone modification to promote altered cellular functional responses to injurious stimuli, even in post-mitotic cells. It is also well known that these epigenetic processes interact with each other along with ATP-dependent chromatin remodeling complexes and non-coding RNAs (miRNAs and lncRNAs), forming a complex regulatory network to facilitate intense regulation of chromatin structure and gene expression. The ability of epigenetic changes to be inherently reversible has always been an attractive concept for therapeutic intervention, yet there are currently no epigenetic-modifying therapies approved or under clinical investigation for the treatment of cardiovascular diseases. Nevertheless, in the context of heart failure, this field remains at an exciting early stage with efforts currently being undertaken to develop more selective agents that target specific epigenetic processes in certain cell types involved in pathological events such as fibrosis and inflammation. Further investigation into the exact involvement of these epigenetic mechanisms specifically in aberrant cardiac remodeling, combined with the development of cell-targeted strategies could potentially lead to the translation of epigenetic modifying therapies for clinical benefit in heart failure.

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Tables

Table 1: Abbreviations

2-HG	2α-hydroxyglutarate	LncRNA	Long non-coding RNAs
5aza	5-azacytidine	LPS	Lipopolysaccharide
5azadC	5-aza-2-deoxycytidine	LV	Left ventricle
5hMeC	5-hydroxymethylcytosine	MBD	Methyl-CpG-binding domain protein
5MeC	5-methylcytosine	MCP-1	monocyte chemoattractant protein-1
AMOTL2	Angiomotin-like protein 2	MDS	Myelodysplastic Syndromes
AngII	Angiotensin-II	MeCP	Methyl-CpG-binding protein
ANCA	Anti-neutrophil cytoplasmic autoantibody	MI	Myocardial infarction
Arg-1	Arginase-1	Mef2	Myocyte enhancer factor-2
ARHGAP24	Rho GTPase activating protein 24	MIP	Macrophage Inflammatory Protein
BMDMs	Bone marrow-derived macrophages	miRNA	Micro RNA
BMP	bone morphogeneic protein	MMPs	Matrix metalloproteinases
BMPER	BMP-endothelial cell precursor-derived regulator	MPPs	Multipotent progenitors
Chaer	Cardiac hypertrophy associated epigenetic reader	Myl7	atrial myosin light chain
ChIP	Chromatin immunoprecipitation	NK	Natural Killer
CLP	Common Lyphoid Progenitor	NT-proBNP	N-terminal pro b-type natriuretic peptide
CMP	Common Myeloid Progenitor	nTreg	natural T-regulatory cells
CPGi	CpG islands	PBMC	Peripheral blood mononuclear cell
COX2	Cycloxygenase-2	PcG	Polycomb group
CpGs	Cytosine-(phosphate)-Guanine dinucleotide	PCM-1	Pericentriolar material-1
CXCL	C-X-C motif chemokine	PCR2	Polycomb repressor 2 complex
DNA	Deoxyribonucleic acid	PDGF	Platelet derived growth factor
DNMT	DNA methyltransferase	PECAM	Platelet endothelial cell adhesion molecule 1
DOT1L	Disruptor of telomeric silencing 1-like	PPAR	Peroxisome proliferator activated receptor
DZNep	3-Deazaneplanocin A	PTIP	PAX interacting with transcription-activation domain) protein 1
ECM	Extracellular Matrix	PTMs	Post Translational Modifications
EMA	European Medicines Agency	RAAS	Rennin-Angiotensin-Aldosterone System
EMT	Epithelial to mesenchymal transition	RASAL1	RAS Protein Activator Like 1
EndMT	Endothelial to mesenchymal transition	RASSF1A	Ras association domain family 1 isoform A

EZH2	Enhancer of zeste homolog 2
FDA	Food and Drug Administration
FHL1	Four-and-a-half LIM domains protein 1
GMPs	Granulocyte-macrophage progenitors
GNAT	GCN4-related N-acetyltransferase
HATs	Histone Acetyltransferases
HDACis	Histone Deacetylase inhibitors
HDACs	Histone Decetyltransferases
HF	Heart failure
HMT	Histone methyltransferase
HSCs	Haematopoietic stem cells
HStCs	Hepatic stellate cells
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF1	Interferon regulatory factor-1
iTreg	Induced Treg cells
JMJDs	Jumonji C-domain containing demethylases
KLF4	Krüppel-like factor 4
KMT	Lysine methyltransferase
KO	Knockout
LDL	Low-density lipoprotein
LINE-1	Long interspersed nuclear element-1
ROS	Reactive Oxygen Species
SAHA	Suberoylanilide hydroxamic acid
SAM	S-adenosylmethionine
SHR	Spontaneously Hypertensive Rat
SIRTs	Sirtuins
SNPs	Single nucleotide polymorphisms
STAT	Signal transducer and activator of transcription
T2DM	Type 2 Diabetes Mellitus
TAB-seq	TET-assisted bisulfite sequencing
TAC	Transaortic Constriction
TET	Ten-eleven translocation
Tfh	T-helper follicular cell
TGF	Transforming Growth Factor
Th	T-Helper
TIMPs	tissue inhibitor of metalloproteinases
TNF	Tumour Necrosis Factor
Tnni	Troponin isoform
Tnnt2	Troponin II
Treg	T-regulatory cell
TrxG	Trithorax Group
TSA	Trichostatin A
VEGF	Vascular Endothelial Growth Factor
WGBS	Whole genome bisulfite sequencing

Table 2: DNA Methylation Changes in Heart failure

Changes in DNA methylation	Heart failure (HF) status/Patient cohort	Genes/elements influenced by changes	Ref
Global promoter hypomethylation & Intragenic hypermethylation	End-stage HF (ischaemic and idiopathic)	DUX4	(230)
Hyper/hypomethylation of gene loci	End-stage HF (ischaemic and idiopathic)	PECAM, AMOTL2, ARHGAP24	(229)
Hyper/hypomethylation of gene loci	Dilated cardiomyopathy	LY75 & ADORA2A	(121)
Global hypomethylation	Ischemic heart disease/stroke	LINE-1	(16)
Global hypomethylation & Reduced DNMT1, DNMT3A and DNMT3B expression	Tetralogy of Fallot	LINE-1	(299)

Table3: Histone Acetylation and Deacetylation in Clinical and Preclinical Models of Heart Failure

Modifying Enzyme	Outcome	Ref
Histone Acetyltransferase		
P300	Increased levels and activity in cardiac biopsies of patients with end stage heart failure (ischemic, dilated or idiopathic)	(353)
	Deficiency of p300 in a knockout mouse model resulted in embryonic lethality with cardiac abnormalities	(380)
Histone Deacetylase		
HDAC1 and HDAC2	Increased levels in both infarcted and non-infarcted myocardium in the LAD ligation model of myocardial infarction (rat; wild type)	(245)
HDAC2	Deficiency of HDAC2 in a knockout mouse model was found to be cardio-protective in response to both pressure overload and β-adrenergic stimulation	(330)
HDAC5	Deficiency of HDAC5 in a knockout mouse model developed non-fatal age related cardiac hypertrophy and an exaggerated hypertrophic response to both pressure overload and overexpression of calcineurin.	(53)
HDAC6	Deficiency of HDAC6 in a knockout mouse model developed a similar aberrant cardiac remodeling in response to AngII infusion but maintained systolic function compared to wild type.	(80)
HDAC9	Deficiency of HDAC9 in a knockout mouse model developed non-fatal age related cardiac hypertrophy and an exaggerated hypertrophic response to both pressure overload and overexpression of calcineurin.	(386)
HDAC5/9	Germline deletion of HDAC5 and HDAC9 resulted in severe embryonic cardiac abnormalities with ventricular septal defects and thin ventricular walls	(53)

Table 4: Role of Sirtuins in Clinical and Preclinical Models of Heart Failure

Sirtuin	Outcome/Findings	Ref
SIRT1	Elevated levels in mice subjected to pressure overload, aging and induction of oxidative stress resulting in cardiac hypertrophy and fibrosis	(7)
	Varying cardiac-specific overexpression resulted in different cardiac phenotypes when mice were subjected to oxidative stress induction and aging. Mild to moderate expression attenuates remodeling and cardiac function whereas high expression promotes cardiomyopathy.	(7)
SIRT3	Deficiency in a knockout mouse model resulted in hypertrophy, fibrosis and cardiac dysfunction in response to aging, AngII and β-adrenergic stimulation.	(123, 313)
	Transgenic expression in mice demonstrated cardio-protective effects in response to aging, AngII and β-adrenergic stimulation.	(313)
SIRT6	Decreased levels found in cardiac biopsies in patients with septal defects, Ischemic, dilated and idiopathic cardiomyopathy.	(316)
	Deficiency in a knockout mouse model results in development of cardiac hypertrophy and fibrosis at 8-12 weeks of age.	(316)
SIRT7	Deficiency in knockout mouse model results in significant cardiac hypertrophy and fibrosis with aging	(331)

Table 5: Histone Methylation in Heart Failure

Cohort/Model Assessed	Methylation Changes	Outcome	Ref
Patient biopsies with Dilated Cardiomyopathy & Dahl Sensitive Rats	Differential H3K4me3 and H3K9me3 profiles	Methylation changes were enriched in genes related to cardiac function	(159)
Cardiac-specific PTIP knockout mouse model subject to isoproterenol and caffeine stimulation	Reduction in H3K4me3	No impact on hypertrophy or fibrosis but induced ventricular arrhythmias	(309)
Cardiac-specific PTIP knockout mouse model subject to pressure overload	Reduction in H3K4me3	Promotes maladaptive remodeling and dysfunction.	(308)
Patient biopsies with ischemic cardiomyopathy or dilated cardiomyopathy	Increased expression of JMJD1A, JMJD2A, and JMJD2B mirrored with decreased H3K9 methylation	Reactivation of Nppa and Nppb and silencing of ATP2A2 and Myh6	(137)
Patient biopsies with hypertrophic cardiomyopathy	Increased levels of JMJD2A	Increased expression of Nppb	(388)
Patient biopsies cardiac hypertrophy	Increased expression of G9a methyltransferase and H3K9me2 in Myh6 promoter	Reduced expression of Myh6	(125)
Mouse model of pressure overload	Decreased levels of H3K4me2 Increased H3K27me3 & H3K36me2 marks and KDM2A levels at the ATP2A2 promoter	Increased expression of Myh7 and reduced expression of Atp2a2	(9)

Decreased levels of H3K9me2 and H3K27me3 marks and

Increased levels of H3K4me2 marks at the Mhy7promoter

Table 6: Role of DNA Methylation in Cardiomyocytes

Enzyme	Associated changes	Ref
DNMT1	Expression is elevated in response to metabolic stress including high glucose, homocysteine and the pollutant, phenanthrene. Associated with reduced miR-133a mediated regulation.	(57)
	Repression of DNMT1 promotes a reduction in global cytosine methylation	(90)
DNMT3A	Suppression results in altered sarcomere structure decreased beat rate variability, impaired calcium signaling and contractility.	(90)
DNMT3B	Deletion resulted in abnormal sarcomeric structure, chamber dilation and reduced contractility under sham conditions but in pressure overload and β-adrenergic stimulation, the hypertrophic response was blunted.	(337)
	Pressure overload induced alternative splicing of Myh7 gene due to differential methylation of three CpGs within intron 27	
DNMT3A/DNMT3B	Knock out of both isoforms in cardiomyocyte did not impair cardiac hypertrophy.	(243)
	Differential gene regulation of Aldh1l1, Krt8 and Sclc9a3 resulting from promoter hypomethylation with enhanced expression in pressure overload.	
TET2	Promotes expression of Myh7 by hydroxymethylation at intergenic enhancer regions	(116)

Table 7: Role of Histone Acetylation and Deacetylation in Cardiomyocytes

Enzyme	Injurious Stimuli	Expression/Activity	Phenotype	Genetic Changes	Ref
P300					
	Phenylephrine	Increased activity	Promotes hypertrophy	Promotes GATA4 binding	(5, 120)
	Pressure overload	Transgenic overexpression	Promotes exaggerated hypertrophy	De novo acetylation of Mef2	(353)
HDAC1	Isoproterenol & Pressure overload	Deficiency of enzyme (conditional KO)	No impact on hypertrophy	No apparent alterations	(222)
HDAC2	No injury	Transgenic overexpression	Promotes hypertrophy	Decreasing expression of Inpp5f activity of Akt, Pdk1 and Gsk3beta.	(330)
	Phenylephrine	Increased expression	Promotes hypertrophy	Reduced expression and binding of KLF4 to the Nppa promoter	(163)
	Isoproterenol	Deficiency of enzyme (conditional KO)	No impact on hypertrophy	No apparent alterations	(222)
HDAC1/2	Postnatal lethality	Deficiency of both enzymes (conditional KO)	Cardiac defects: dilated cardiomyopathy and arrhythmias	Altered expression of genes involved in myofibrillar proteins (Tnni1 and Tnni2) and specific calcium channel subunits (CACNA1H and CACNA2D2).	(222)
HDAC3	No injury	Deficiency of enzyme (conditional KO)	Hypertrophy with alterations in myofibril and	Aberrant expression of genes involved in cardiac metabolism (UPC2, UPC3, FACS, GLUT4)	(223)

			mitochondrial structure		
	Isoproterenol	Transgenic overexpression	Increased proliferation at birth but showed similar hypertrophic response as WT	Reduction of cyclins, CDKs, and E2F transcription factors and up-regulation of the negative regulators of cell cycle (Cdkn1a, Cdkn1b, Cdkn1c, and Cdkn2c)	(329)
SIRT1	No injury	Transgenic over-expression	Promotes hypertrophy	Deacetylation and increased activity of Akt1	(314)
SIRT3	Phenylephrine & Angiotensin-II	Increased expression	Promotes myocyte survival	Deacetylation of Ku70 preventing Bax-mediated apoptosis	(315)
SIRT6	No injury	Deficiency of enzyme (conditional KO)	Promotes hypertrophy	Enhanced H3K9 acetylation and promoted binding and transcriptional activity of c-Jun	(316)
	Isoproterenol & Pressure overload	transgenic over-expression	Prevented hypertrophic response	SIRT6 deacetylates H3K9 and prevents activation of IGF-signaling related genes	(316)

Table 8: Changes in Histone Methylation in Cardiomyocytes

Histone Modifying Enzyme	Methylation changes	Role in myocyte	Ref
Histone Methyltransferase			
DOT1L	Promotes H3K79me2 marks	Involved in cardiomyocyte differentiation and maturation	(252)
G9a	Promotes H3K9me2 methylation	Transcriptional silencing of Myh6 in pressure overload by recruiting DNMTA	(125)
EZH2	Promotes H3K27 methylation	Prevents expression of hypertrophic genes and can be inhibited by interaction with the lncRNA Chaer	(349)
KMT2B/KMT2C	Promotes H3K4me3 methylation	Important in cardiomyocyte response to injury. Regulates expression of ADRA1A, ADRA1B, JUN, ATP2A2, ATP1A2, SCN4B, and CACNA1G in pressure overload.	(309, 310)
Histone Demethylase			
JMJD1A	Demethylates H3K9me1 and H3K9me2	Upregulated in order to compensate for knockdown of JMJD2A	(137)
JMJD2A	Demethylates H3K9me2 and H3K9me3	Involved in promoting cardiac hypertrophy by binding to FHL1 promoter and recruiting myocardin and serum-response factor	(388)
JMJD1A/2A	Demethylation of H3K9 methylation	Prevents expression of hypertrophic genes	(137)

Table 9: DNA Methylation in Fibroblasts

Modifying Enzyme	Response to activation	Effect on fibrosis	Associated changes	Ref
DNMT1	Increased levels in hypoxia	Induction of profibrotic markers αSMA and Col1.	Global hypermethylation	(273, 350)
DNMT3A	Increased levels in hypoxia	EndMT of human coronary endothelial cells.	Promoter methylation of RASAL1.	(366, 367)
	Increased levels after PDGF treatment	Increased proliferation and levels of αSMA and Col1.	Regulates expression of RASSF1A.	(325)
DNMT3B	Increased levels in hypoxia	Induction of profibrotic markers αSMA and Col1.	Global hypermethylation.	(350)
TET3	Suppressed in response to TGFβ	Induction of pro-fibrotic markers.	Promoter methylation of RASAL1 due to impaired TET3-demethylation.	(321)

Table 10: Acetylation and Deacetylation in Fibroblasts

Modifying Enzyme	Association with fibrosis	Ref
p300	Elevated levels are associated with enhanced collagen expression elicited by Smad-dependent TGFβ activation.	(33)
HDAC1 and 2	Increased levels in ischemic myocardium.	(245)
	Treatment with Class I HDAC inhibitor (Mocetinostat) reduced expression of αSMA, Col III, and MMP2.	
HDAC4	Knockdown resulted in inhibition of TGFβ-induced αSMA expression, associated with regulation of Akt phosphorylation.	(110, 119)
	Regulates expression of MMP9 and MMP13.	(266)
HDAC6	siRNA knockdown impaired TGFβ-induced αSMA expression.	(110)
HDAC8	siRNA knockdown impaired TGFβ-induced αSMA expression.	(110)

Table 11: Histone Methylation and Demethylation in Fibroblasts

Modifying Enzyme	Role in fibroblasts	Ref
Histone Methyltransferase		
G9a	Induction of expression in response to TGF-Smad3 promoting a fibrotic phenotype.	(145)
	Associated with increased H3K9me1 levels.	(145, 201)
EZH2	Involved in COX2 repression by H3K9me3.	(69)
	Promotes PPARγ silencing by H3K27me3 on 3' exons.	(205)
ASH1	Promotes expression of αSMA, TIMP-1, collagen-1 and TGFβ through addition of H3K4me3 marks.	(254)
Histone Demethylase		
KDM2B	Regulator of fibroblast function.	(151)

Table 12: DNA Methylation in Immune Cells

Cell type	Impact/Outcome	Ref
Haematopoetic stem cell	Reduced DNMT1 activity prevents generation of lymphoid progenitor cells.	(39)
	Loss of DNMT3A and DNMT3B prevents cellular self-renewal and impairs differentiation.	(52)
	Tet2 is a negative regulator of HSC differentiation as loss promotes differentiation to the monocyte/macrophage lineage.	(172)
Neutrophils	Hypomethylation of transcription factors (PU.1 and GFI1) and granular proteins (myeloperoxidase, elastase, and proteinase 3) associated with neutrophil differentiation.	(276)
Macrophage	Macrophages isolated from hind limb ischemia and metabolic dysfunction model have differential promoter methylation promoting a pro-inflammatory M1 phenotype.	(15)
	Persistent differential methylation changes in monocytes in Type1 diabetic patients. Hypomethylation of TXNIP was validated in monocytes exposed to high concentrations of glucose.	(59)
	DNMT1 and DNMT3B expression is increased in adipose tissue macrophages from *ob/ob* mice and is associated with a pro-inflammatory phenotype associated with increased methylation of the PPARγ promoter.	(345, 379, 381)
	Reduced promoter methylation at the LXR and PPARγ is associated with athero-protective effects.	(46)
	Hypermethylation of SOCS1 results in prolonged secretion of LPS induced TNFα and IL-6 secretion.	(60)
Dendritic cell	Altered expression of DNMT1, DNMT3A and TET2 during monocyte to dendritic cell differentiation. This was mirrored by dynamic changes in methylation and expression of SRC, PLEKHG6 and ITGB2.	(389)
	CpGs in the promoter region of CD209 become hypomethylated during differentiation of monocytes to dendritic cells.	(41)

	The IL-4-JAK3-STAT6 pathway is important in regulating TET2 demethylation for differentiation of monocytes to dendritic cells.	(334)
	DNA hypomethylation, increased 5hmC and altered gene expression occur in response to infection with Mycobacterium tuberculosis.	(251)
T cells	DNMT1 is involved along with the other DNMTs to varying degrees to maintain the Cd4 locus in a silenced state in CD8+ T cells.	(293)
Th1	Th1 cell acquisition is associated with demethylation at the IFN-γ gene locus.	(289)
Th2	DNMT3A is recruited to the IFN-γ promoter and rapidly methylates the IFN-γ promoter during Th2 polarization.	(155)
Th17	Th17 cells have marked demethylation at CpG regions in IL17A, Zfp362, Ccr6, Acsbg1, Dpp4, Rora and Dclk1 genes.	(375)
Memory T cell	Differential methylation between memory and naïve CD4+ cells facilitating inflammatory gene expression (including IL-17A, IL-13 and IFNγ) upon stimulation in memory cells.	(175)
Treg	Methylation in a conserved CpG-rich region upstream of FoxP3 promoter can influence stability of its expression in Treg cells.	(167)
	Involved in silencing FoxP3 expression in NK cells.	(399)

Table 13: Histone Acetylation and Deacetylation in Macrophages

Modifying Enzyme	Role	Ref
Acetyltransferase		
p300	Promotes expression of IFNA, TNFα and IL-6 in response to viral infection.	(92)
Deacetylase		
HDAC3	Suppresses IL-4 target gene expression by binding to PU.1 enhancer regions.	(234)
	Deficiency facilitates acquisition of an M2 phenotype enhanced expression of Arg1, Clec7a, Ym1, and Retnla	(234)
	Overexpression is seen in macrophages in atherosclerotic plaques and promotes expression of IL-6.	(136)
	Promotes expression of IFNβ.	(58)
HDAC5	Overexpression promoted expression of MCP-1 and TNFα.	(259)
HDAC6	Promotes LPS-induced proinflammatory phenotype	(371)
HDAC7	Increased expression found in thioglycolate-elicited peritoneal macrophages. HDAC7 isoform interacts with HIF-1α to promote proinflammatory gene expression.	(296)
HDAC9	Deficiency impaired LPS-induced gene expression , improved cholesterol efflux, skewed to the M2 phenotype with increased acetylation at the ABCA1, ABCG1, and PPAR-γ gene promoters	(45)

Table 14: Histone Acetylation and Deaceylation in Treg Cells

Modifying Enzyme	Role	Ref
Acetyltransferase		
p300	Promotes Treg survival and suppressive activity	(197)
Histone Deacetylase		
HDAC3	Inhibits NFkB activation and IL2 expression in Treg cells.	(342)
HDAC6	Involved in suppressing FoxP3 through deacetylation as Treg cells from HDAC6 deficient mice exhibit increased expression of IL-10 and FoxP3 compared to wild type.	(28, 76)
HDAC9	Impairs Treg function through regulation of HSP70 and inhibition of STAT5 transcriptional activity.	(29, 77)
HDAC6/Sirt1	Combined deficiency of these improved preservation and increased proliferation of FoxP3+ Tregs	(29)

Figure Legends

Figure 1: Schematic overview highlighting epigenetic changes associated with cardiac injury that lead to the acquisition of hyperactive cellular phenotypes in cardiomyocytes, fibroblasts and immune cells, and promote aberrant pathological remodeling in the heart.

Figure 2: *Regulation of gene transcription by DNA methylation and demethylation in the promoter region*. The addition of a methyl group (-CH3) to carbon 5 on the cytosine ring (C), is catalysed by the DNA methyltransferase (DNMT) enzymes forming 5-methylcytosine (5MeC). S-adenosylmethionine (SAM) is utilized as a methyl donor that is subsequently converted to S-adenosylhomocysteine (SAH) upon donation. Methyl-CpG-binding proteins (MeCP) and methyl-CpG-binding domain protein (MBD) proteins are recruited to 5MeC resulting in gene silencing by preventing transcription factor (TF) and binding of RNA polymerase II (RNApolII) binding. Conversely, removal of methylated cytosines can be enzymatically carried out by the ten-eleven translocation (TET) enzymes, which oxidize 5MeC to 5-hydroxymethylcytosine (5hMeC). Small molecule inhibitors of DNMT activity prevent 5MeC formation and together these processes promote gene expression by allowing TF and RNApolII to bind to the DNA.

Figure 3: Control of gene expression through regulation of chromatin structure by acetylation and methylation of histone proteins. Formation of heterochromatin structure and repression of gene expression is facilitated by both the removal of acetyl groups from histone tails by histone deacetylases (HDACs) and the actions of histone lysine methyltransferases (HMTs) and demethylases (HDMs) forming repressive histone marks (H3K9, H3K20, H3K27). Conversely, promotion of gene expression and euchromatin structure is carried out by enzymatic addition of acetyl groups by histone acetyltransferases (HATs) and the actions of HMTs and HDMs forming active histone marks (H3K4, H3K26, H3K79).Abbreviations: HMTs: Histone Methyltransferases HDMs: Histone Demethylases, HATs: Histone Acetyltransferases, HDACs: Histone Deacetylases, CH3: methyl group. Yellow pentagon: Active histone marks, Blue pentagon: Repressive histone marks, Green marks: active acetylation marks

Didactic Figure Legends

Figure1: Teaching Points: This illustrated figure demonstrates that various types of injury to the heart can drive epigenetic changes in cardiomyocytes, fibroblasts and immune cells. These epigenetic changes can result in the acquisition of aberrant phenotypes which drive pathological remodeling in the heart which ultimately leads to the development of heart failure.

Figure 2: Teaching Points: This illustrated figure highlights the regulation of gene expression by DNA methylation (gene repression) and demethylation (gene activation) at the promoter region of a gene.

Figure 3: Teaching Points: This illustrated figure facilitates understanding of gene regulation by modifications of the amino-terminal tails of histone proteins, focusing on specifically the modifications acetylation and methylation. It also highlights the active and repressive histone marks that are associated with active gene expression or gene silencing.

Further Reading

Cross-References

Cardiac fibroblast physiology and pathology

Cardiovascular responses to stress

Cellular basis of physiological and pathological myocardial growth (legacy)

Intracellular signaling of cardiac fibroblasts

Myocardial cell signaling during the transition to heart failure

Fetal programming and cardiovascular pathology

General cardiac response to injury

Obesity and nutrition effects on cardiac structure and function

Pathophysiology of heart failure

Wound healing

Epigenetics and environmental stressors

Supplementary Information

N/A

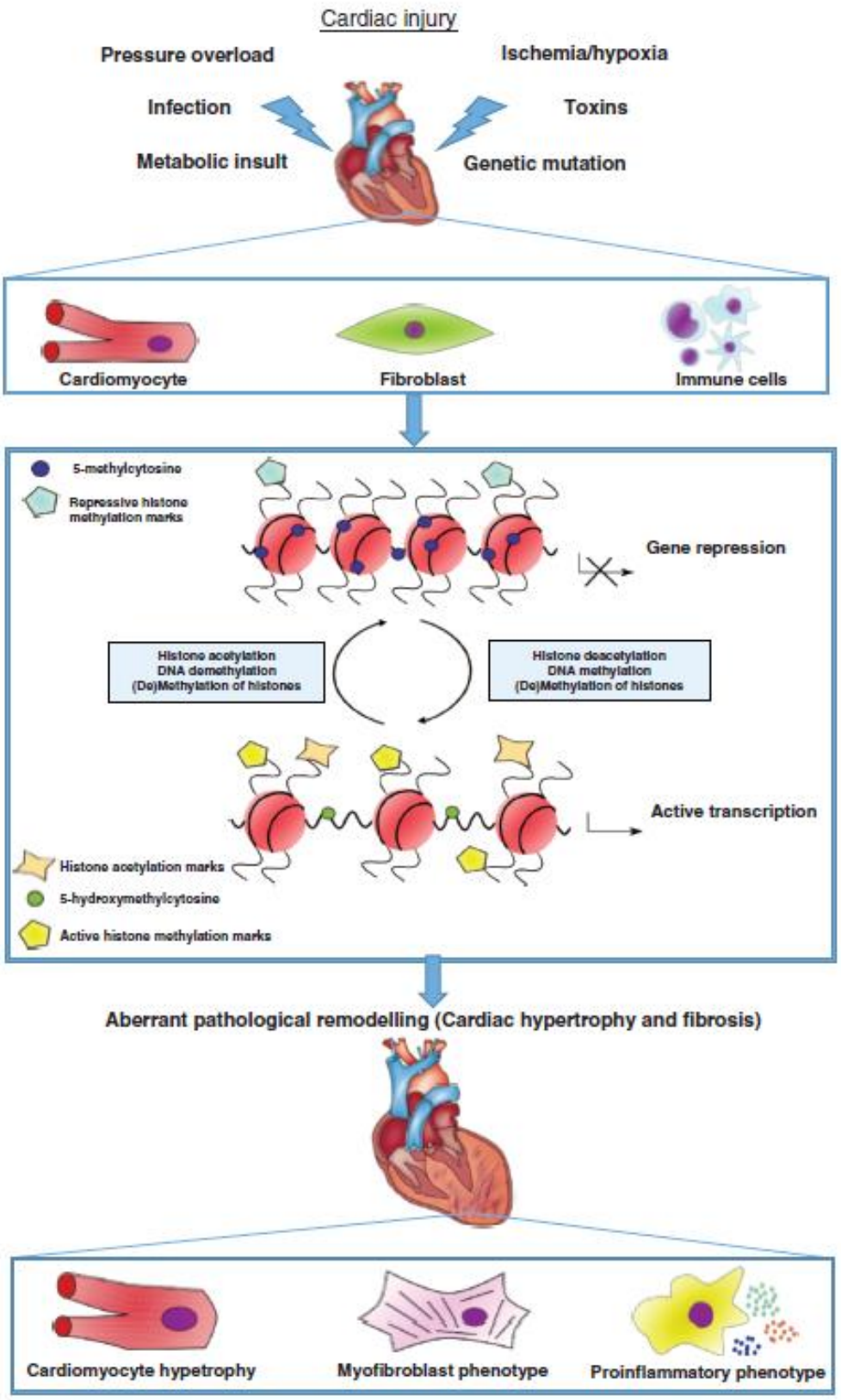

Figure 1 Schematic overview highlighting epigenetic changes associated with cardiac injury that lead to the acquisition of hyperactive cellular phenotypes in cardiomyocytes, fibroblasts and immune cells, and promote aberrant pathological remodeling in the heart.

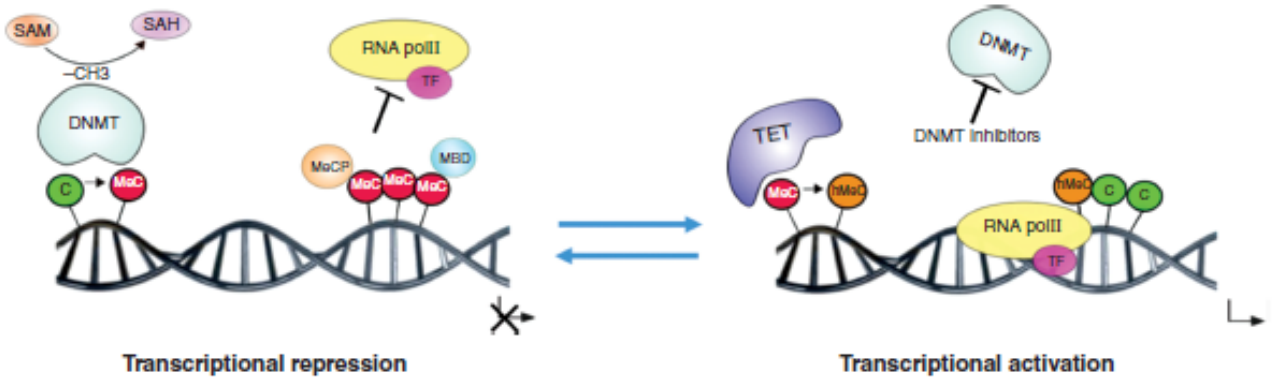

Figure 2 *Regulation of gene transcription by DNA methylation and demethylation in the promoter region.* The addition of a methyl group (-CH3) to carbon 5 on the cytosine ring (C), is catalysed by the DNA methyltransferase (DNMT) enzymes forming 5-methylcytosine (5MeC). S-adenosylmethionine (SAM) is utilized as a methyl donor that is subsequently converted to S-adenosylhomocysteine (SAH) upon donation. Methyl-CpG-binding proteins (MeCP) and methyl-CpG-binding domain protein (MBD) proteins are recruited to 5MeC resulting in gene silencing by preventing transcription factor (TF) and binding of RNA polymerase II (RNApolII) binding. Conversely, removal of methylated cytosines can be enzymatically carried out by the ten-eleven translocation (TET) enzymes, which oxidize 5MeC to 5-hydroxymethylcytosine (5hMeC). Small molecule inhibitors of DNMT activity prevent 5MeC formation and together these processes promote gene expression by allowing TF and RNApolII to bind to the DNA.

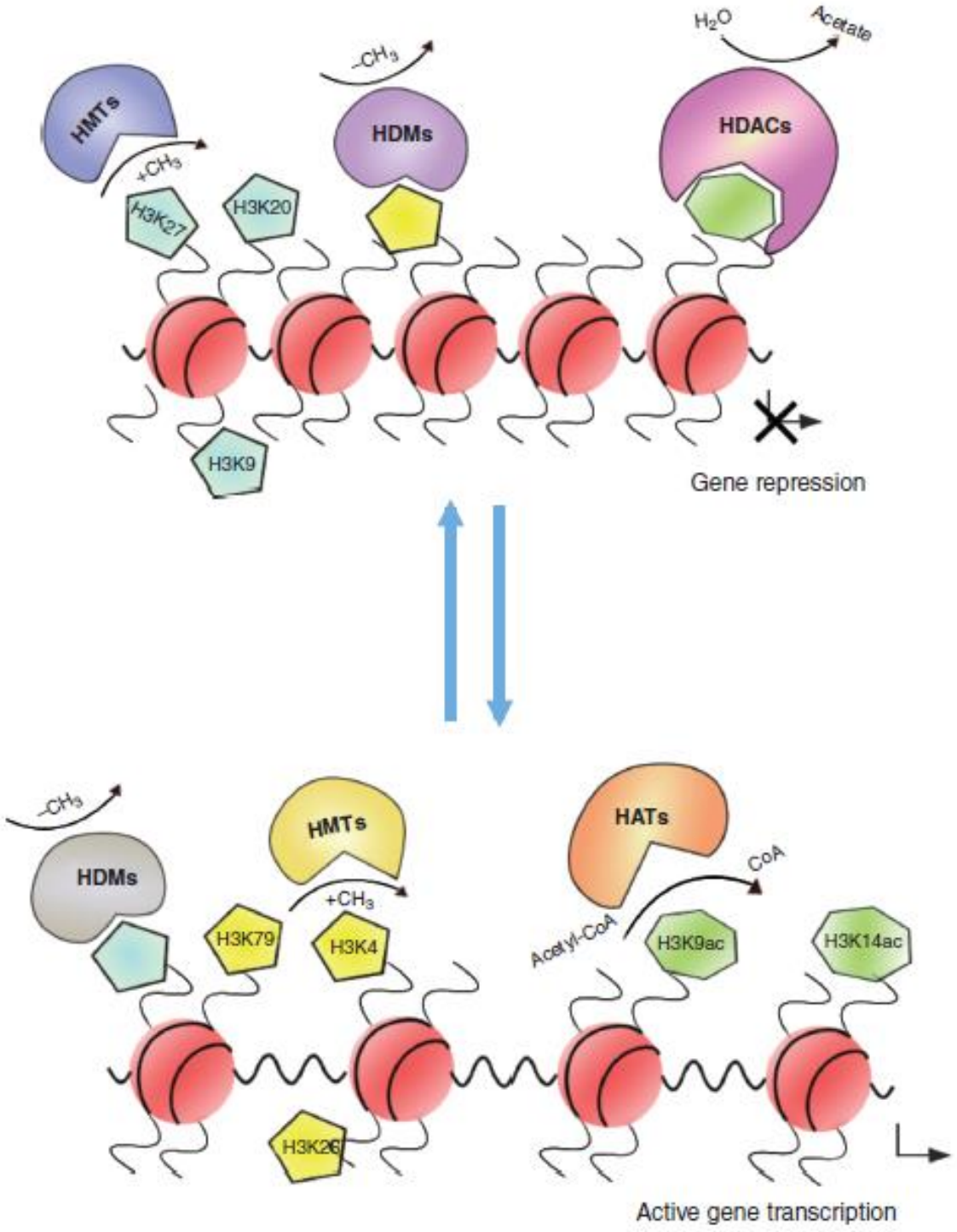

Figure 3 *Control of gene expression through regulation of chromatin structure by acetylation and methylation of histone proteins.* Formation of heterochromatin structure and repression of gene expression is facilitated by both the removal of acetyl groups from histone tails by histone deacetylases (HDACs) and the actions of histone lysine methyltransferases (HMTs) and demethylases (HDMs) forming repressive histone marks (H3K9, H3K20, H3K27). Conversely, promotion of gene expression and euchromatin structure is carried out by enzymatic addition of acetyl groups by histone acetyltransferases (HATs) and the actions of HMTs and HDMs forming active histone marks (H3K4, H3K26, H3K79). Abbreviations: HMTs, histone methyltransferases; HDMs, histone demethylases; HATs, histone acetyltransferases; HDACs, histone deacetylases; CH3, methyl group. Yellow pentagon: active histone marks, blue pentagon: repressive histone marks, green marks: active acetylation marks.